Optimization of surveillance of Bovine Viral Diarrhea in Danish dairy herds

Foddai, Alessandro

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Optimization of surveillance of Bovine Viral Diarrhea in Danish dairy herds

Ph.D. Thesis

Alessandro Foddai

2014

DTU Veterinærinstituttet

Optimization of surveillance of Bovine Viral Diarrhea in Danish dairy herds

Alessandro Foddai

1870 Frederiksberg C

VET-PHD-2014

This thesis was prepared in the

Section for Epidemiology at the

National Veterinary Institute

Technical University of Denmark (DTU-VET)

Bülowsvej 27, 1870 Frederiksberg C

Copenhagen, Denmark

Phone: + 45 27499397

alefo@vet.dtu.dk

www.vet.dtu.dk

Ph.D. study number 102812

The photos in the front page were obtained from http://commons.wikimedia.org/wiki/

Supervisors

Main supervisor:

Peter Lind*

Senior Adviser

Section for Epidemiology

National Veterinary Institute

Technical University of Denmark

Co-supervisor:

Tariq Halasa

Senior Researcher

Section for Epidemiology

National Veterinary Institute

Technical University of Denmark

Co-supervisor:

Åse Uttenthal

Professor

Section for Virology

National Veterinary Institute

Technical University of Denmark

*Between July 2010 and June 2013, Dr. Claes Enøe was the main supervisor.

Assessment committee

Professor Lars Erik Larsen (Chairman)

Section for Virology, National Veterinary Institute (DTU-VET)

Professor Hans Houe

Department of Large Animal Sciences, University of Copenhagen (KU-SUND)

Dr. Ann Lindberg

Head of the Swedish Zoonosis Centre at the Department of Disease Control and Epidemiology, National Veterinary Institute (SVA), Uppsala

"The optimal strategy will always be based on estimates which will have a degree of uncertainty…..treat the optimum as a guide rather than a fixed rule that must be obeyed"

(Cannon, 2009)

Table of contents

Summary

This thesis comprises studies on surveillance of Bovine Viral Diarrhea (BVD) in Danish dairy herds. BVD is caused by a *Pestivirus* of the *Flaviviridae* family (BVDV) that can infect domestic and wild ruminants (e.g. deer). The main sources of infection are the persistently infected animals (PI) which shed BVDV during all life, while transiently infected (TI) animals only shed the virus for a short time period in small amounts compared to PIs. BVD is considered to be distributed worldwide and although its course is usually subclinical, outbreaks can have an important impact on animal health and income of farmers.

In Denmark, the BVD eradication program started in 1994. During the last twenty years, while the BVD herd incidence decreased to only sporadic cases, the average herd size has increased. Currently (2014), BVD is considered eradicated from Denmark. In this situation, newly infected dairy herds (e.g. after import of infected cattle) could be more difficult to detect compared to the past, due to the lower prevalence of antibody positive milking cows and the (expected) higher dilution of antibodies in bigger milk containers. Therefore, an evaluation and an eventual optimization of the BVD surveillance system in Danish dairy herds were considered necessary by the Danish Cattle Federation.

In study I, we verified how the BVD herd prevalence, the herd size and the dilution of individual milk within the bulk tank milk (BTM) changed, between 2003 and 2010. Moreover, the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997) and the SVANOVIR ELISA (Juntti at al., 1987; Niskanen, 1993) were compared on milk and serum samples. The prevalence of antibody positive milking cows, which can be detected by each of those tests, was estimated by diluting positive individual milk and making artificial milk pools. We found that the median herd size increased noticeably during the investigated years, whereas the prevalence of BVDV infected dairy herds decreased from 0.51% to 0.02%, together with the BTM antibody levels in the National dairy population. We also found that the SVANOVIR ELISA could detect a lower prevalence of antibody positive cows compared to the Danish blocking ELISA (0.78% vs. 50%). Hence, the former could detect newly infected herds shortly after infection when only few

milking cows have seroconverted in the herd. In blood, the two tests performed similarly. Thus both ELISAs can be used to test serum (e.g. in imported live cattle).

In study II, a stochastic simulation model was developed in R and was validated using field data from an infected herd. Using this model the Danish blocking ELISA, the SVANOVIR ELISA and the indirect ELISA BVD/MD p80 Institute Pourquier (Beaudeau et al., 2001a) were compared regarding their BVD detection time in different herd sizes. The SVANOVIR ELISA appeared to give the fastest response and so, was the test of preference for an early-warning surveillance system where infected herds are detected as soon as possible by BTM testing.

In study III, the risk of introducing BVD from abroad into Danish cattle dairy herds was assessed per year and per trimester. Imports of live cattle, semen, embryos, truck visits, use of vaccines and veterinarians and hoof trimmers practicing across borders were considered as possible routes of BVDV introduction. The main source of infection was represented by the import of live cattle from countries where BVD is endemic. With the current situation, the overall median risk was estimated to one BVDV introduction per 9 years (5^{th} percentile = 59; 95^{th} percentile = 3). By introducing simple measures of risk mitigation, such as testing all imported animals and always disinfecting the tools used abroad for hoof trimming, the risk can be reduced to one introduction per 33 years (200; 8).

Finally in study IV, the temporal sensitivity (*SSe*) of the current Danish surveillance system (based on BTM testing with the blocking ELISA) was evaluated, according to the information obtained in studies I, II, and III and using stochastic scenario trees (Martin et al., 2007a). Additionally, the confidence in complete freedom (*PFree*) from BVD in Danish dairy herds (< 1 infected herd) and the confidence (*PLow*) in low herd prevalence (<0.02% infected herds) were estimated. BVDV introductions from abroad, e.g. due to import of a PI calf or a TI milking cow were taken into account. Moreover, alternative surveillance strategies were considered. These were (i) using the SVANOVIR ELISA on BTM and (ii) testing dairy herds at higher risk of BVDV introduction (importing live cattle) in individual serum and other dairy herds in BTM. From a general point of view, the temporal *SSe*, the *PLow* and the *PFree* were higher testing at 365 days from BVDV introduction, than testing at 90 days. Estimates were usually higher for the

SVANOVIR than for the blocking ELISA, and when a PI calf rather than a TI cow was introduced to the herd(s). Hence, if the SVANOVIR ELISA was used to test BTM samples, the temporal *SSe* would be increased together with the related *PFree* and *PLow*. Testing individual blood in herds importing cattle would not increase the temporal *SSe* noticeably, due to the very low number of dairy herds which import live animals during a year period (only 8/4109 herds in 2010).

Resumé

Denne afhandling består af undersøgelser vedrørende overvågning af bovin virus diarré (BVD) i danske malkekvægsbesætninger. BVD er forårsaget af en pestivirus (BVDV) af familien af Flaviviridae, der kan inficere husdyr og vilde dyr (f.eks rådyr). Den vigtigste smittekilde er persistent inficerede dyr (PI) som udskiller BVDV hele livet, mens forbigående inficerede (TI) dyr udskiller virus i en kort periode og i små mængder i forhold til PI. BVD anses for at have global forekomst, og selv om dens forløb normalt er subklinisk, kan udbrud i en besætning give en betydelig forringelse af dyresundheden og landmandens indkomst.

I Danmark startede et BVD-udryddelsesprogram i 1994. Dette er i væsentlig grad baseret på analyse af ad specifikke antistoffer i tankmælk. I løbet af de sidste tyve år er den gennemsnitlige besætningsstørrelse steget markant, mens sygdomsforekomsten er faldet til kun få sporadiske tilfælde. I øjeblikket (2013) anses BVD for udryddet fra Danmark. I denne situation kunne nysmittede malkekvægsbesætninger (f.eks. efter import af inficeret kvæg) være vanskeligere at opdage i forhold til tidligere, på grund af lavere forekomst af antistof positive malkekøer og højere fortynding af antistoffer i større mælkebeholdere. Derfor vurderer landbrugets organisationer (Dansk Kvæg) at en evaluering og en eventuel optimering af BVD overvågningssystemet er nødvendig.

I studie I opgjorde vi, hvordan BVD prævalensen i besætningerne, besætningernes størrelse og fortyndingen af individuelle mælk i tankmælken (BTM) blev ændret mellem 2003 og 2010. Desuden blev den danske blokerende ELISA (Rønsholt et al., 1997; Bitsch et al., 1997) og SVANOVIR ELISA (Juntti at al., 1987, Niskanen, 1993) sammenlignet på mælke- og serumprøver. Prævalensen af antistofpositive malkekøer, der kan påvises ved hver af disse test, blev estimeret udfra fortynding af positiv individ-mælk og kunstige mælkepuljer. Vi fandt, at den mediane besætningsstørrelse steg mærkbart gennem de undersøgte år, mens prævalensen af BVDV inficerede malkekvægsbesætninger faldt fra 0.51% til 0.02%, samtidigt med et fald i BTM antistof niveauet i den nationale kvægpopulation. Vi fandt også at SVANOVIR ELISA'en kunne påvise en lavere prævalens af antistof-positive køer forhold til den danske blokerende ELISA (0.78% versus 50%). Førstnævnte kunne altså påvise nyligt inficerede besætninger på et

tidligere tidspunt d..v.s. når kun få malkekøer havde serokonverteret. Udført på blod er de to tests ækvivalente, og begge kan bruges til at teste serum fx i importeret levende kvæg.

I studie II blev en stokastisk simulationsmodel udviklet i R og valideret ved anvendelse af feltdata. Ved hjælp af denne model blev den danske blokerende ELISA, SVANOVIR ELISA'en og en indirekte ELISA BVD / MD-P80 Pourquier Institute (Beaudeau et al., 2001a) sammenlignet med hensyn til tiden for deres påvisning af BVD ved forskellige besætningsstørrelser. SVANOVIR ELISA-testen viste sig at være den hurtigste, og er derfor at foretrække som BTM test i et overvågningssystem med tidlig varsling, d.v.s. hvor inficerede besætninger identificeres så hurtigt som muligt.

I studie III blev risikoen for at indføre BVD fra udlandet til danske malkekvægsbesætninger vurderet årligt pr trimester. Import af levende kvæg, sæd, embryoner, lastbilbesøg, brug af vacciner samt af klovbeskærere og dyrlæger, der praktiserer på tværs af grænserne, blev undersøgt som mulige smitteveje for BVDV introduktion. Den vigtigste smittekilde viste sig at være import af levende kvæg fra lande, hvor BVDV er endemisk. I den nuværende situation blev den samlede mediane risiko estimeret til én BVDV introduktion pr. 9 år (5. percentil = 59, 95-percentilen = 3). Ved at indføre enkle foranstaltninger til risikoreduktion, såsom testning af samtlige importerede dyr og konsekvent desinfektion af værktøjer, der anvendes til klovbeskæring, kan risikoen reduceres til én introduktion pr. 33 år (200, 8).

Endelig blev i studie IV den tidsmæssige følsomhed (SSe) af det nuværende danske overvågningssystem (BTM baseret testning med blokerende ELISA) evalueret med inddragelse af oplysninger indhentet i studie I, II og III og ved hjælp af stokastisk scenarieanalyse (Martin et al., 2007a). Derudover blev sikkerhedsgrænsen for fuldstændig frihed (PFree) for BVD i danske malkekvægsbesætninger (<1 inficeret besætning) og sikkerhedgrænsen for lav prævalens (PLow) (<0.2% inficerede besætninger) anslået. Forskellige typer BVDV introduktion fra udlandet, fx import af en PI kalv eller en kortvarigt inficeret malkeko (TI) blev taget i betragtning. Desuden blev alternative overvågningsstrategier overvejet, dels (i) testning med SVANOVIR ELISA'en på BTM og dels (ii) testning af serumprøver i malkekvægsbesætninger med høj risiko for BVDV introduktion (import af levende kvæg). Generelt er den tidsmæssige SSe,

PLow og PFree højere ved 365 dage fra BVDV introduktion end 90 dage efter, og estimaterne er normalt højere for SVANOVIR end for blokerende ELISA samt når en PI kalv snarere end en TI blev introduceret til besætningen. Ved anvendekse af SVANOVIR ELISA'en til testning af BTM prøver, ville den tidsmæssige SSe kunne optimeres sammen med de tilknyttede PFree og PLow. Testning af individuelle blodprøver i importerende kvægbesætninger ville ikke øge den tidsmæssige SSe mærkbart på grund af det meget lave antal af malkekvægsbesætninger, der importerer levende dyr (kun 8/4109 i 2010).

Sommario (in Italian)

Questa tesi comprende studi sulla sorveglianza epidemiologica della diarrea virale bovina (BVD) in allevamenti danesi di bovine da latte. La BVD è causata da un *Pestivirus* della famiglia *Flaviviridae* (BVDV) in grado di infettare i ruminanti domestici e selvatici (per esempio, il cervo). Le principali fonti d'infezione sono gli animali persistentemente infetti (PI) che diffondono il virus della BVD durante tutta la vita, mentre gli animali transitoriamente infetti (TI) diffondono il virus per tempi brevi e in piccole quantità rispetto agli animali PI. La BVD è considerata una malattia a diffusione cosmopolita e anche se il suo decorso è di solito subclinico, i focolai possono avere un importante impatto sulla salute degli animali e il reddito degli allevatori.

In Danimarca, il programma di eradicazione della BVD è iniziato nel 1994. Nel corso degli ultimi venti anni, mentre l'incidenza della malattia è diminuita (limitata a soli pochi casi sporadici), il numero medio di capi presenti nelle aziende da latte è aumentato significativamente. Attualmente (2014), la BVD è considerata debellata dalla Danimarca, ma in questa situazione, negli allevamenti da latte appena infettati (per esempio dopo l'importazione di bovini infetti da altri paesi) potrebbe essere più difficile rilevarla, a causa della bassa prevalenza di vacche siero positive dentro le aziende, e a causa della diluizione più elevata degli anticorpi individuali nei contenitori di latte a capacità maggiore. Pertanto, una valutazione e l'eventuale ottimizzazione del sistema di sorveglianza erano considerati necessari da parte dell´ Associazione Nazionale Allevatori (Danish Cattle Federation).

Nello studio I, abbiamo verificato come la prevalenza di aziende infette, la dimensione delle aziende da latte danesi e la diluizione del latte individuale all'interno del latte di massa (BTM) sono cambiati, tra il 2003 e il 2010. Inoltre, l´ ELISA usata in Danimarca, chiamata Danish blocking ELISA (Rønsholt et al, 1997; Bitsch et al., 1997) e la SVANOVIR ELISA (Juntti at al., 1987; Niskanen, 1993) usata in altri paesi scandinavi (per esempio in Svezia), sono state confrontate su campioni di latte e siero proveninenti da anziende infette. La prevalenza di bovine sieropositive in lattazione, alla quale il test usato classifica l´ azienda come infetta, è stata stimata testando latte diluito di vacche siero positive e pools artificiali di latte. Abbiamo rilevato che la dimensione delle aziende è aumentata notevolmente nel corso degli anni esaminati,

mentre la prevalenza di allevamenti infetti è calata da 0.51% a 0.02%, insieme ai livelli anticorpali nel tank di azienda. Abbiamo anche riscontrato che la SVANOVIR ELISA e´ in grado di rilevare una prevalenza di vacche sieropositive piu´ bassa, rispetto all´ ELISA danese (0.78% vs 50%). Quindi, per l'attuale situazione, la SVANOVIR ELISA potrebbe rilevare gli allevamenti infetti subito dopo l'infezione, quando solo poche vacche in lattazione sono immunizzate dentro la mandria. Nei campioni di sangue, le due ELISA hanno fornito performance simili. Quindi entrambi i test posson essere usati per testare il siero (per esempio di bovini importati).

Nello studio II, un modello di simulazione stocastica è stato sviluppato in R ed è stato convalidato con dati di campo provenienti da una azienda recentemente infetta. Usando questo modello la Danish blocking ELISA, la SVANOVIR ELISA e l´ ELISA BVD/MD p80 Istitut Pourquier (Beaudeau et al., 2001a) sono state confrontate in mandrie di diverse dimensioni, per quanto riguarda il loro tempo di rilevamento della malattia. La SVANOVIR ELISA e´ risultata significativamente più “veloce”, e quindi puo´ esser considerata il test di preferenza per un sistema di sorveglianza e di allarme rapido.

Nello studio III, il rischio di introdurre il virus della BVD dall'estero è stata quantificato su base annuale e trimestrale. Le importazioni di bovini vivi, di dosi di seme, di embrioni, le visite di camion usati all´ estero, l'uso di vaccini, le visite di veterinari e le visite di trimmers degli unghioni che praticano a livello transfrontaliero, sono stati considerati come possibili vie di introduzione del BVDV. La principale fonte di infezione è risultata essere l'importazione di bovini vivi da paesi in cui la BVD è endemica. Con la situazione attuale, il rischio medio è stato stimato pari a una introduzione ogni 9 anni (5° percentile = 59; 95° percentile = 3). Il rischio può essere ridotto ad una introduzione ogni 33 anni (200, 8), con l'uso di semplici misure di mitigazione, come l´ analisi obbligatoria del sangue per tutti gli animali importati e la disinfezione degli strumenti utilizzati all´ estero per il trimming degli unghioni.

Infine, nello studio IV, la sensibilità temporale (*SSe*) dell'attuale sistema di sorveglianza danese (basato sull´ uso della Danish blocking ELISA sul latte del tank) è stata valutata, in base alle informazioni ottenute negli studi di I, II, e III; ed usando i cosi´ detti “alberi di scenario stocastici” o “stochastic scenario trees” (Martin et al., 2007a). Inoltre, sono state stimate la

confidenza in completa indennita´ (“freedom”, *PFree*) da BVD negli allevamenti da latte danesi (<1 allevamento infetto) e la confidenza (*PLow*) in una bassa prevalenza di aziende infette (<0.02% allevamenti infetti). Introduzioni di BVDV dall'estero, per esempio a causa dell´ importazione di un vitello PI o di una bovina TI in lattazione, sono state prese in considerazione. Inoltre, sono state considerate strategie di sorveglianza alternative. Queste erano: (i) utilizzare la SVANOVIR ELISA su tutti i tank del latte presenti in Danimarca, e (ii) testare nel siero individuale le aziende che importano bovini vivi (aziende a piu´ alto rischio) mentre le rimanenti aziende son testate nel latte del tank. Da un punto di vista generale, la *SSe* temporale, la *Plow* e la *PFree* sono risultate più elevate testando a 365 giorni anziche´ a 90 giorni dall´ introduzione del virus in azienda. Le stime erano generalmente più elevate per la SVANOVIR che per la Danish blocking ELISA, e quando un vitello PI (piuttosto che una vacca TI) è introdotto nella mandria (o in piu´ mandrie). Quindi, la SVANOVIR ELISA potrebbe esser utilizzata per testare i campioni del tank. In tal modo la *SSe* temporale potrebbe essere ottimizzata insieme alle relative *PLow* e *PFree*. Testare nel sangue individuale le mandrie che importano bovini, non aumenterebbe la *SSe* temporale in modo significativo, a causa del bassissimo numero di allevamenti da latte che importano bovini (solo 8/4109 aziende nel 2010).

Preface

This PhD project was financed by the Ministry of Food, Agriculture and Fisheries of Denmark (Grant number 3412-09-02603) and by the Danish Cattle Federation. The main target was to evaluate and eventually optimize the surveillance of Bovine Viral Diarrhea in Danish dairy herds and to propose a new general approach, through which surveillance of cattle diseases can be routinely evaluated and optimized in Denmark.

During the last eight years of my carrier (2006-2014) I had the pleasure to study in different European universities where I improved my knowledge on animal production systems, veterinary epidemiology and disease surveillance. After I took the veterinary degree at the Veterinary University of Sassari (Italy), I decided to take a master on European animal production systems at the University of EI-Purpan (France) and a master degree on Animal Health Management at the University of Wageningen (The Netherlands).

Then I decided to start this PhD in order to continue improving my skills. During the last four years I learned a lot by working in contact with several experts, stakeholders and colleagues. I got many answers to the research questions I had at the beginning of the project, and I tried to help to improve the surveillance of cattle diseases in Danish dairy herds.

The direct result from this PhD project is a surveillance approach that combines optimized samples collection and data analysis with an adequate utilization of laboratory diagnostics on BTM samples. BVD was used as a model disease. The output includes an “operational diagram”, through which surveillance of emerging diseases can be routinely evaluated and optimized.

Frederiksberg, October 2014.

Acknowledgments

First of all I would like to thank my father, mother and brother (Mario, Franca and Costanzo) for supporting me during my studies in different European countries and during this Ph.D. project. You have been great supporters and you helped me to keep up the good spirit to reach my goals in different stages of my education.

Next, I would like to thank my first two supervisors, Prof. Åse Uttenthal and Claes Enøe (who followed my studies in the first part of the project), for selecting me four years ago and for giving me their trust. Dear Prof. Åse, it has been a pleasure to work with you in the laboratories of Lindholm (the remote island of Denmark :-). I think you have been very patient with me and I have learned a lot from you. Dear Claes, we had very inspiring discussions and you helped me to see the challenges of the project from different perspectives. I have improved my skills and I am happy to have known you.

I am also very thankful to Peter Lind, who followed my studies in the last year and half of my Ph.D. Dear Peter, it has been a pleasure to have discussions with you, and many times, I thought you know more than Google ;-). I also acknowledge Tariq Halasa so much for being my supervisor, especially in developing the stochastic model in R. I think you have all the characteristics to be a very good teacher and supervisor for many other students.

I thank my co-authors Anette Boklund, Anders Stockmarr and Kaspar Krogh for giving me very useful suggestions and for supporting me during the studies.

I thank my colleagues at DTU-VET (Section for Epidemiology), Carsten Kirkeby, Kaare Græsbøll, Mette Ely Fertner, Mariann Chriél, Rene Bødker, Birgit Kristensen, Larry Paisley, Erik Jacobsen, Ana Carolina Lopes Antunes, Per Kantsø Nielsen, Camilla Birkegård and Nils Toft, for creating such a nice working environment and for carrying out interesting scientific discussions. For Rene, a little special remark: apart from some sporadic cases where you won, you always lost playing the billiard, but I really appreciated your continuous spirit of revenge. I think you improved a lot…. well done ☺.

For the others who contributed to the completion of my Ph.D. I would like to thank Erik Rattenborg, Jørgen Nielsen, and Esben Jakobsen (from the Danish Cattle Federation). You have been very professional and efficient in giving me all the information, to put me in contact with the different stakeholders of the Danish dairy industry and to give me samples and datasets. It has been a pleasure to work with you.

From the laboratories of Lindholm I thank Grith J. Clausen and Frank Hansen for helping me with the lab work.

Last, but not least, I thank the Ministry of Food, Agriculture and Fisheries of Denmark, the Danish Cattle Federation for funding the project, and the DTU-VET for giving me the opportunity to work at the Section for Epidemiology.

Appendix

I. Alessandro Foddai, Claes Enøe, Anders Stockmarr, Kaspar Krogh, Åse Uttenthal, 2014. Detection of antibodies against Bovine Viral Diarrhoea Virus in bulk milk according to herd size and antibody ELISA used. *Submitted to Acta Veterinaria Scandinavica.*

II. Alessandro Foddai, Claes Enøe, Kaspar Krogh, Anders Stockmarr, Tariq Halasa, 2014. Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. *In press at Preventive Veterinary Medicine.*

III. Alessandro Foddai, Anette Boklund, Anders Stockmarr, Kaspar Krogh, Claes Enøe, 2014. Quantitative assessment of the risk of introduction of bovine viral diarrhea virus in Danish dairy herds. *Preventive Veterinary Medicine 116, 75-88.*

IV. Alessandro Foddai, Anders Stockmarr, and Anette Boklund, 2014. Evaluation and optimization of the temporal surveillance system sensitivity (SSe) for Bovine Viral Diarrhea in Danish dairy herds. *Submitted to Preventive Veterinary Medicine.*

Aims of the thesis

The aims of the thesis were to:

(1) Compare different ELISA tests in detection of antibodies against BVDV in milk and serum samples (manuscript I).

(2) Estimate the lag time between BVDV introduction in a herd (through PI or TI animals) and detection of antibodies in BTM, for different ELISAs and herd sizes (manuscript II).

(3) Quantify the risk of introduction of BVDV into Danish dairy herds (manuscript III)

(4) Evaluate the present BVD surveillance system for Danish dairy herds and give advice on how to optimize the system (manuscript IV).

Outline of the thesis

Chapter 1, is a general introduction to the disease, its pathogenesis, epidemiology, available diagnostic tests and control strategies. Moreover, general approaches of surveillance are described, and the different eradication phases for BVD in Denmark are reported.

In chapter 2, the main research questions are given for each aim of the thesis, together with the respective materials and methods used and the results. The latter are reported as "Answers" to the Danish Cattle Federation.

In chapter 3, a discussion of the main results is carried out.

In chapter 4, the main conclusions are synthesized, while in Chapter 5 the challenges and limitations of the studies are reported.

Finally, in chapter 6, future perspectives are discussed and a final operational diagram is proposed.

List of abbreviations:

Abbreviation	Meaning
AC-ELISA	Antigen-capture ELISA
ARRj	Adjusted relative risk of infection within the "*j*" risk category
BHP	Between herds prevalence
bl%	Blocking percentage (value for the Danish blocking ELISA)
BRSV	Bovine respiratory syncytial virus
BTM	Bulk tank milk
BTMCSe	Temporal sensitivity for the surveillance component based on BTM testing
BTMSSe	Temporal surveillance system sensitivity when all dairy herds in the country are tested in BTM
BVD	Bovine viral diarrhea
BVDV	Bovine viral diarrhea virus
CP	Cytopathic BVD biotype
EBL	Enzootic bovine leucosis
ELISA	Enzyme linked immunosorbent assay
$EPI_{ImpoCattle}$	Effective probability of infection within the *ImpoCattle* category
$EPI_{NoImpoCattle}$	Effective probability of infection within the *NoImpoCattle* category
FA	Fluorescent antibody staining
HRP	High risk period (N.B. in section 1.5 this means Horseradish peroxidase)
IBR	Infectious bovine rhinotracheitis

IHC	Immonohistochemical staining
ImpoCattle	Dairy herds which import live cattle
NCP	Non-cytopathic BVD biotype
NoImpoCattle	Dairy herds which do not import live cattle
NPV	Negative predictive value of the surveillance system
OD	Optical density (e.g. for the Danish blocking ELISA)
PAnim	Stochastic scenario tree used in manuscript III, to estimate the probability of introducing BVDV into Danish dairy herds by imported live animals
PEmb	Stochastic scenario tree used in manuscript III, to estimate the probability of introducing BVDV into Danish dairy herds by imported embryos
PFree	Confidence in complete freedom from BVD (P_H < 0.02% or <1/4109 infected herds)
P_H	Between-herds design prevalence
PI	Persistently infected cattle
PIntro	Overall probability of BVDV introduction in Danish dairy herds
PI-3V	Parainfluenza-3 virus
PLow	Confidence in low between-herds design prevalence (P_H < 0.2% or < 8/4109 infected herds)
PP	Percent positivity value (SVANOVIR ELISA)
PriorPInf	Prior probability that the country is infected at the assumed between-herds and within-herd design prevalence, at the beginning of the surveillance period
$PrP_{ImpoCattle}$	Proportion of dairy herds which import live cattle
$PrP_{NoImpoCattle}$	Proportion of dairy herds which do not import live cattle
PSem	Stochastic scenario tree used in manuscript III, to estimate the probability of introducing BVDV into Danish dairy herds by imported semen

PTR	Probability that the threshold/design prevalence (P_U) is reached on the day the herd is tested
PTrim	Stochastic scenario tree used in manuscript III, to estimate the probability of introducing BVDV into Danish dairy herds by hoof trimmers practicing in Denmark and abroad
$PTR_{ImpoCattle}$	Probability that the threshold/design prevalence is reached in the *ImpoCattle* herds on the day of sampling
$PTR_{NoImpoCattle}$	Probability that the threshold prevalence is reached in the *NoImpoCattle* herds on the day of sampling
PTruck	Stochastic scenario tree used in manuscript III, to estimate the probability of introducing BVDV into Danish dairy herds by trucks used abroad
P_U	Design prevalence within the milking group (testing strategies "a" and "b"), or overall within herd design prevalence (testing strategies "c" and "d")
RBS	Risk based surveillance
$RR_{ImpoCattle}$	Relative risk of infection in the risk category *ImpoCattle*
$RR_{NoImpoCattle}$	Relative risk of infection in the risk category *NoImpoCattle*
RT-PCR	Reverse transcriptase-polymerase chain reaction
Se	Test sensitivity
SerumCSe	Temporal sensitivity of the surveillance component based on individual serum testing (for *ImpoCattle* herds in surveillance strategy "c" and "d")
Sp	Test specificity
SSC	Surveillance system component
SSe	Temporal surveillance system sensitivity
SSp	Surveillance system specificity
TI	Transiently infected cattle
VI	Virus isolation test
WHP	Within herd prevalence

CHAPTER 1

Introduction

1.1 Bovine Viral Diarrhea Virus (BVDV)

Bovine Viral Diarrhea (BVD) is a disease, which affects domestic (Olafson et al., 1946) and wild ruminants, e.g. deer (Haigh et al., 2002). It is caused by a single-stranded RNA *Pestivirus* (BVDV) of the *Flaviviridae* family. BVDV is closely related to Classical Swine Fever (CSFv) and Border Disease viruses (BDv) (Collett et al., 1988; Peterhans et al., 2010), and is represented by two main species, BVDV-1 and BVDV-2 (Pellerin et al., 1994; Ridpath et al., 1994), though recently, discussion arose over the emergence of a new BVDV species (BVDV-3, atypical or ´HoBi´-like bovine pestiviruses) (Ståhl et al., 2007; Liu et al., 2009; Decaro et al., 2011; Larska et al., 2012). Within each species different isolates with biological and antigenic diversity can be found. The BVDV-1 species is composed of 11 phylogenetic subgroups (1a-1k), while BVDV-2 subgroups are classified into 2a and 2b (Becher et al., 1999; Vilček et al., 2001). Moreover, BVDV can be classified in two biotypes: cytopathic (CP) and non-cytopathic (NCP), according to the damage caused in cell cultures (Corapi et al., 1988; Peterhans et al., 2010). Only NCP biotypes are considered capable of being transmitted between animals, while CP biotypes are only isolated in animals with Mucosal Disease (MD) (see below). Brownlie at al. (1987) argued that a more likely mean of origin of the cytopathic virus is from within the infected herds by viral mutation of the NCP to the CP biotype. This observation was suggested by circumstantial evidence from field observations, though it was not proven in the laboratory.

1.2. Pathogenesis, clinical signs and immunity

When susceptible non immune dams are exposed to the BVD virus (and become transiently infected or TI) during the first 120 days of pregnancy, the calf becomes immunotolerant and persistently infected (PI) with BVDV (McClurkin et al., 1984). PI animals shed the virus in large amounts in their body excretions for the rest of their lives (Brownlie et al., 1987; Baker, 1990). Moreover, PI cows will give birth to PI calves (McClurkin et al., 1984). Thus, the most important role in the spread of the BVDV is played by the PI animals.

The uterine infection can also cause abortions, stillbirths or weak calves, reduced fertility and early embryonic loss (McClurkin et al., 1984; Brownlie et al., 1987; Moennig and Liess 1995; Fray et al., 2000). BVDV-2 strains are more often isolated from animals with hemorrhagic syndrome (HS) and thrombocytopenia (Ridpath et al., 1994; Pellerin et al., 1994). Therefore, clinical forms of BVD can vary in severity according to moment of infection (early vs. late pregnancy), and BVDV species.

PI calves infected with NCP strains may seem healthy, but can develop a clinical syndrome called Mucosal Disease (MD) and usually die within two years of age (Brownlie et al., 1987; Houe, 1993; Loehr et al., 1998). The mucosal disease can appear as "early onset" within 2-3 weeks, when animals carrying a NCP strain are also infected with a CP strain (super-infection). or "late onset" MD (e.g. several months after super-infection) (Fritzemeier et al., 1997; Loehr et al., 1998). Some authors suggested that the virus found in animals with late onset MD can be derived from a genetic recombination between the persistent NCP and the superinfecting CP BVD viruses (Fritzemeier et al., 1997). In animals with MD, erosions and ulcers can be observed in the oral mucosa and in the intestinal tract (Fritzemeier et al., 1997). Loehr et al. (1998), reported that PI animals carrying NCP virus and superinfected with a CP strain, can have three main phases of MD (Phase I, II and III). In phase I, PI animals could develop clinical signs (of varying degrees) within three weeks from superinfection, and some could die within this period (early onset MD) suffering from a watery-bloody diarrhea. In Phase II, an asymptomatic period of 32-45 days could be observed. After that period, animals could develop fever and late onset MD (Phase III) with hemorrhages, hemoglobinuria, hematuria and death.

Moreover, there are indications that BVDV is a synergistic agent, especially with other pathogens which can affect the respiratory tract (Graham et al., 1998; Fulton et al., 2000). For instance, Fulton et al. (2000) found that BVDV infections could occur in conjunction with other infections, especially with *Pasteurella haemolytica*, parainfluenza-3 virus (PI-3V) and bovine respiratory syncytial virus (BRSV). The role of BVDV in mixed pathogen infections is considered important, because it is immunosuppressive. In fact, BVDV causes a decrease in the numbers of B and T lymphocytes (Bolin et al., 1985) and in the lung macrophage function (Welsh et al.,

1995). On the other hand, Houe and Heron (1993) found that PI calves had an adequate immune response to various types of antigenic stimuli (e.g. antitoxin response after tetanus immunization and response to tuberculin skin test after immunization against paratuberculosis). This response was not significantly different from that of the control calves.

Transiently infected animals may show mild clinical symptoms (e.g. fever and/or drop in the milk yield), but most infections (70-90%) are subclinical (Ames, 1986; Baker 1990). TI animals start shedding the virus in small amounts (compared to PIs) 4-7 days after exposure and remain viremic for 10-14 days. TI animals seroconvert within 2-3 weeks from infection and become lifelong immune to BVDV (Brownlie et al., 1987; Baker, 1990; Fredriksen et al., 1999).

Immunity due to colostral antibodies can last till 6-8 months of age (Kendrick and Franti, 1974; Coria and McClurkin, 1978). In PI animals a small antibody response can be observed if they are exposed to strains, which are different from those that caused the PI status (Fritzemeier et al., 1997; Loehr et al., 1998).

It is also usually accepted that immune cows are able to protect the fetus from infection (Duffell et al., 1984).

In contrast, when immunization is raised by use of vaccines, complete protection of the fetus is difficult to achieve, especially if killed vaccines are used (Coria and McClurkin, 1978; van Oirschot et al., 1999). Xue et al. (2009) showed that when a Modified-Live virus vaccine (MLV) was injected in pregnant heifers, the fetal infection rate could be reduced by 82% for BVDV-Type 1 and by 75% for BVDV Type 2. On the other hand, there is a general concern when live BVDV vaccines are used, or when live vaccines are utilized to immunize cattle against other pathogens (e.g. BHV-1), because accidental contaminations with wild type BVDV could occur and could cause BVD outbreaks within the herd (Barkema et al., 2001; Antonis et al., 2004).

1.3. Transmission and resistance in the environment

Transmission of BVDV between animals within a herd can happen by different routes. Those are: direct contact between susceptible and viremic (PI or TI) animals (Brownlie et al., 1987), contact with contaminated equipment (e.g. due to lochia and fetal fluids eliminated during birth or abortion of a PI calf) (Lindberg et al., 2004), and airborne transmission up to 40 meters from PI animals (Mars et al., 1999; Bitsch et al., 2000). The transmission rates reported in the literature for PI animals are by far higher than for TIs (see manuscript II; Viet et al., 2004; Ezanno et al., 2007). TIs spread BVDV in smaller amounts and for shorter periods than PIs, and thus, they are less effective to transmit the BVDV to other susceptible animals (Meyling et al., 1990; Niskanen et al., 2000). Hence, for example, when BVDV spread is simulated within a herd, it is usually assumed that TI animals spread the virus only within their group, while PIs can spread the virus between different animal groups (Viet et al., 2004; Ezanno et al., 2007).

BVDV transmission between herds and between countries could occur by infectious animals introduced to the herd (PI or TI), by cows carrying PI calves (Trojan cows) (Lindberg and Alenius, 1999; Fray et al., 2000; Lindberg et al., 2001), by contaminated live vaccines (Barkema et al., 2001; Antonis et al., 2004), semen (Niskanen et al., 2002), embryos (Stringfellow and Givens 2000; Gard et al., 2010), and by contaminated medicines (Niskanen and Lindberg, 2003; Katholm and Houe, 2006)

Risk of BVDV transmission from wild animals (e.g. roe deer) to cattle is usually considered to be very low (Lindberg et al., 2006). Sheep with antibodies to BVDV may be found, probably due to contact with cattle (Tegtmeier et al., 2000; Uttenthal et al., 2005).

Under natural conditions, the infectious dose needed to cause viremia in susceptible animals is difficult to define (e.g. for the spread of BVDV by contaminated materials). Nevertheless, in experimental trials naïve animals can show viremia, if challenged intra-nasally with an aerolized NCP BVDV type 1 and type 2 at doses of 2.5 X 10^6 TCDI (50% tissue culture infectious dose) and 1.0 X 10^6 TCDI, respectively (Xue et al., 2009).

Especially in small closed herds, the BVDV spread can die out (self-clearance) in the case no new BVDV introductions occur in the herd. The probability of self-clearance within a herd is dependent on the immune status of the herd (immune animals do not give birth to PI calves), the frequency of removing PI calves, the herd size and the herd structure (Lindberg and Alenius, 1999; Lindberg and Houe, 2005).

In the environment, the virus has similar resistance to that of the Classical Swine Fever Virus. Such resistance is dependent on the pH and temperatures. For instance, Depner et al. (1992) reported that the virus can survive 151 hours at pH 4 and temperature 4°C.

1.4 Diagnosis

As reviewed by Larson et al. (2005) several diagnostic methods can be used to detect virus positive animals. Those are the virus isolation technique (VI), the Antigen-capture ELISAs (AC-ELISA), the reverse-transcriptase polymerase chain reaction (RT-PCR) (Zimmer et al., 2004, Larson et al., 2005), the fluorescence antibody staining (FA), and the immunohistochemical (IHC) staining (Ellis et al., 1995). On the other hand, to distinguish between PI and TI animals, each of those methods should be applied twice 3-4 weeks apart. In case viremia is detected only in the first test, the animal is TI. If the second test gives a positive result as well, the animal can be classified as PI.

Virus isolation (VI) can be done from serum and other tissues, or using bovine cell cultures (e.g. kidney) inoculated with test specimens (Ellis et al., 1995; Zimmer et al., 2004; Larson et al., 2005), and studied by immunofluorescence few days later. If samples are not optimal (e.g. due to autolysis) these techniques have low sensitivity (Ellis et al., 1995). Moreover, cell culturing facilities are required and false negatives are possible, when serum is tested in the presence of maternal antibodies (Zimmer et al., 2004).

Antigen-capture ELISAs are cheap and easy to perform. Those can be used on serum, plasma, or skin samples. At the same time, analysis for BVDV antigen detection requires samples of

good quality and it can be negatively affected by the presence of maternal antibodies in serum of young calves (Zimmer et al., 2004)

RT-PCR assays have high sensitivity and moderate costs if pooled samples are used. Moreover, this test is suitable for identification of PI animals even when maternal antibody titers due to colostrum are high (Zimmer et al., 2004). In Denmark, a RT-PCR is used (Uttenthal et al., 2005; Rasmussen et al., 2007) to find PI calves in herds with positive BTM.

Immunohistochemical (IHC) staining of skin biopsy (e.g. ear notches samples for viral antigen detection), is usually highly sensitive to find PI animals and to make BVDV diagnosis in cases of abortion and neonatal death (Ellis et al., 1995). The latter authors found that the IHC test has *Se* and *Sp* 97% in the diagnosis of BVDV-induced bovine abortion or neonatal death, while the VI test showed 83% *Se* and 100% *Sp*. For the FA those estimates were 77% and 83%, respectively. On the other hand, the IHC test is laboratory intensive.

For antibody detection, the virus neutralization test (VNT) is usually considered the reference test (Edwards, 1990; Beaudeau et al., 2001a; Beaudeau et al., 2001b; Houe et al., 2006). Nevertheless, VNT is laborious and expensive so antibody ELISA systems are usually used for BVD surveillance. Moreover, different BVD species and strains can be detected by using antibody ELISAs (Rønsholt et al., 1997; www.svanova.com).

Several antibody ELISAs are used in Europe. Those are usually blocking ELISA (Rønsholt et al., 1997; Kramps et al., 1999; Beaudeau et al., 2001b; Beaudeau et al., 2001c) or indirect ELISA systems (Juntti et al., 1987; Beaudeau et al., 2001a). The main difference between the two systems is that, in a blocking (or competitive) ELISA, antibodies present in the sample bind with the antigen added by the lab technician and prevent binding between the antigen and the agent-specific enzyme-conjugated antibody. In the indirect ELISA (non-competitive) the specific antibodies present in the sample bind to the antigen immobilized on the ELISA plate and then they are detected by an enzyme-conjugated immunoglobulin-specific antibody. For further details on advantages and drawbacks of the different ELISA systems we refer to Schrijver and Kramps (1998).

In this project, a blocking and an indirect ELISA have been used and compared in the laboratories of Lindholm (Denmark) in milk and serum (see manuscript I). These were the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997) and the indirect ELISA SVANOVIR®BVDV-Ab (Juntti et al., 1987; Niskanen et al., 1989; Niskanen et al., 1991, Niskanen, 1993). In manuscript II, also the indirect ELISA BVD/MD p80 Institut Pourquier was considered (Beaudeau et al., 2001a).

1.5 Antibody ELISAs used for BVD surveillance in Scandinavia

The Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997) used in Denmark is a liquid-phase blocking ELISA where the milk or serum is added together with the standardized antigen in a well pre-coated by porcine IgG anti-pestivirus. Antigen bound by antibodies in the sample are washed away, while remaining antigen will be bound by the porcine IgG anti-pestivirus, and subsequently detected by Horseradish peroxidase (HRP) conjugated-rabbit antibody towards BVDV. The higher the amount of antigen bound to the coated well the higher is the optical density (OD) read by the ELISA reader. The positivity of the samples is measured calculating the blocking % (bl%) caused by the presence of antibodies against BVDV in the sample. In each well, the result is equal to:

bl% = 100 – [*(OD negative control - OD sample)/ OD negative control*]*100

In serum, the estimated *Se* and *Sp* are 96.5% and 97.5% respectively, if a cut-off bl% of 50 is used (Rønsholt et al., 1997). The *Se* and *Sp* for individual milk have never been estimated.

The SVANOVIR ELISA (Juntti et al., 1987; Niskanen et al., 1989; Niskanen et al., 1991, Niskanen, 1993) is used in other countries (e.g. Sweden). This is an indirect ELISA where pre-coated BVDV and control wells are incubated with milk or serum overnight. In our studies, milk samples were tested undiluted, but serum was diluted 1:10 with PBS-Tween buffer before the analysis. The next day HRP conjugated anti-cow antibodies were used to induce staining of positive wells. Results of the test were obtained as percent positivity values (PP). The PP were calculated as:

PP= 100[(OD sample- OD negative sera) / (OD positive sera – OD negative sera)]*

In individual milk, the estimated *Se* and *Sp* are 95.2% and 100% respectively (cut-off PP = 9%), while in serum the *Se* is 100% and the *Sp* is 98.2% (cut-off PP = 15%) (www.svanova.com).

1.6 Geographical distribution of BVD

BVD is considered to be distributed worldwide (Meyling et al., 1990; OIE, 2004), and countries with strong cattle and/or semen trade links, are likely to have the same BVDV strains (Vilček et al., 2001).

In 2001, Vilček and colleagues typed 78 BVDV isolates coming from different EU countries. Between those, 76 were BVDV-1 and two were BVDV-2 strains (Vilček et al., 2001). Hence BVD-2 strains are less frequently isolated in Europe. An outbreak due to BVDV-2 was reported in The Netherlands due to use of a live attenuated vaccine for infectious bovine rhinotracheitis (IBR) contaminated with BVDV (Barkema et al., 2001; Antonis et al., 2004).

In countries where BVD is endemic the prevalence of persistently infected cattle is usually around 1-2% (Houe and Meyling, 1991; Houe, 1999). The prevalence of transiently infected cattle within a herd is rarely reported in the literature and according to Billinis et al. (2005) can reach 14% (95%CI: 11-18%). This figure could vary considerably, in contrast to the prevalence of PI animals, because of the transient and sometimes fluctuating behavior of the infection within a herd where there is a PI.

Moreover, in endemic countries, a high prevalence (e.g. 35, 65%) of herds infected with PIs can be found (Paton et al., 1998; Zimmer et al., 2002), and 60-85% of the cattle can be antibody positive (Houe, 1999).

BVD is considered to be eradicated in Scandinavian countries. In Lower Austria and Switzerland, eradication programs have been launched and the prevalence of infected herds reduced markedly (Rossmanith et al., 2010; Presi et al., 2011).

In Denmark and Sweden sporadic herd infections have been observed in 2010 and 2011, with few dairy herds infected (Ståhl and Alenius 2012). In those years, the national dairy population was almost similar in the two countries. In Denmark, in 2010, approximately 4100 dairy herds were present. In Sweden, during the same year, Alvåsen et al. (2012) reported that 4252 dairy herds were present (without counting herds with less than 20 cows and herds with more than 40 dead cows per 100-cows years).

1.7 Veterinary surveillance systems

According to the Terrestrial Animal Health Code (OIE, 2011), surveillance is: "the systematic ongoing collection, collation, and analysis of information related to animal health and the timely dissemination of information to those who need to know, so that action can be taken". Surveillance systems can have different purposes, such as early detection of an infectious agent, demonstrating freedom from disease (e.g. in a country), or studying disease epidemiology (e.g. prevalence). Sensitivity (*Se*), specificity (*Sp*), timeliness, and efficiency of the surveillance system must be evaluated properly (Thurmond, 2003), because those determine the acceptance, or refusal, of the surveillance system at national or international level.

Veterinary services worldwide are required to exchange and harmonize surveillance data in a transparent way (Hoinville et al., 2013). At the same time, the diagnostic test/s used and the sampling strategy can create some challenges in the implementation of any surveillance plan, because they affect the amount of resources needed, e.g. sample size and monetary budget. High costs are one of the main constraints to surveillance. Moreover, surveillance systems can be based on sampling all herds present in the country (as is the case in Danish dairy herds), or by applying random sampling, and/or targeting population strata at higher risk of infection to reduce the sampling costs (Hadorn and Stärk, 2008; Schuppers et al., 2010; Blickenstorfer et al., 2011).

To find a balance between veterinary needs and surveillance sustainability, an approach defined as “risk based surveillance” (RBS) has been proposed (Stärk et al., 2006, Mintiens and Zagmutt, 2006).

RBS systems have been defined as “those in the design of which exposure and risk assessment methods have been applied together with traditional design approaches in order to assure appropriate and cost-effective data collection” (Stärk et al., 2006). Within this context, “efficiency” is interpreted as the amount of surveillance information obtained relative to amount of resources invested, while the term “efficacy” (or effectiveness) indicates the capacity of the surveillance system to comply with the requisites for which it was set up, e.g. high sensitivity and high confidence in disease freedom (Stärk et al., 2006; Mintiens and Zagmutt, 2006).

Moreover, as stated by Cannon (2002) the basis to claim freedom from disease is to prove that sufficient testing is done, and the final result will be a certain probability that the disease, if present in a country or area, will occur at a lower level than a predefined prevalence in the national herd (Cameron and Baldock, 1998). This “theoretical” prevalence, that can be determined by previous studies (made to substantiate freedom from infection) or by the legislation, is usually referred as the design prevalence (or P*) (Martin et al., 2007a; Martin et al., 2007b; Martin, 2008). We called it “theoretical” prevalence, because it expresses an ambition to demonstrate with a certain confidence (e.g. ≥ 95%) that if the disease is present in the country, this occurs with a percentage of infected herds/animals, which is less than or equal to the P*. The combination of design prevalence and the desired confidence level will influence the sample size. The lower the design prevalence, the larger the sample size needed to detect the disease in a country or area, with high confidence.

The required sample size using a RBS system can be significantly lower than that in more traditional surveillance approaches (e.g. based on simple random sampling), because the population strata at higher risk of infection may be targeted (Hadorn and Stärk, 2008; Schuppers et al., 2010). By doing so, the probabilities of detection can be increased, because

the sampling is addressed to “where the disease is most likely to be present” and costs of the sampling scheme can thus be reduced.

The main phases of RBS could be synthetized as:

a) Defining whether the disease/hazard requires priority of surveillance compared to other diseases/hazards. This step is sometimes considered one of the characteristics, which differentiates targeted surveillance from risk based surveillance (Stärk et al., 2006; Stärk, 2009). In fact, according to Stärk (2009): “Targeted surveillance is not a synonym, but rather a special case of risk based surveillance when sampling is conducted in high-risk populations. Risk based surveillance is a broader concept that can also involve priority setting at higher levels. For example, risk assessment can be used to select pathogens that should be included in a surveillance program”. This phase is also known as “risk based prioritization”, where hazards are selected for surveillance based on information about the probability of their occurrence and the extent of biologic and/or economic consequence of their occurrence (Hoinville, 2011). Hence, in that case, the word “risk” is used as it is used in the field of risk analysis to include both the probability that the hazard occurs as well as the consequence of the occurrence (while usually in epidemiology, risk refers to the probability of occurrence). Nevertheless, this phase is not needed if for example, the surveillance of some pathogen is made compulsory by policy makers at international level, to keep free trades between countries (e.g. for African Swine Fever).

b) Defining the risk based sampling, so that the sampling strategy is designed to reduce the cost or enhance the accuracy of the surveillance system by preferentially sampling strata within the target population, that are more likely to be exposed, affected, detected, become affected, transmit infection or cause other consequences (e.g. large economic losses or trade restrictions)(Hoinville, 2011).

c) Defining the sampling frequency and sample size according to the sensitivity (Se) of the test/s used, the design prevalence (P*) and the risk of infection in the different population strata, with the objective to reach a high confidence to detect the pathogen, if it is present in

the country at the assumed P*. Such a confidence is represented by the surveillance system sensitivity (*SSe*) (Martin et al., 2007a)

d) Evaluating the sensitivity of the entire surveillance system (*SSe*) and making a comparison with the previous surveillance approaches. So called "stochastic scenario-tree models" can be used to give an evaluation of the RBS program, its components and *SSe* (Martin et al., 2007a; Martin et al., 2007b; Martin, 2008). Then, a simple comparison between the previous and the risk based surveillance systems can be made by calculating the sensitivity ratio, which is given by SSe_{RBS} /$SSe_{current}$. In case a value higher than 1 is obtained the RBS strategy would give an improvement of the *SSe* (Martin et al., 2007a).

Finally, the RBS systems could be used, to estimate the confidence in freedom from infection at country/area level, according to (1) the prior probability (*PriorPInf*) that the country/area is infected (at the beginning of the surveillance period) at the assumed between-herds design prevalence P_H and within-herd design prevalence P_U, (2) the probability (*PIntro*) that the pathogen/infection is introduced from abroad during the surveillance period, (3) the risk of infection across the population strata and (4) the *SSe* estimated in step "d" (Martin et al., 2007a).

1.8 The scenario tree framework to evaluate veterinary surveillance systems

A technical evaluation of the entire surveillance system and its *SSe* can be made by applying the scenario-tree modeling framework that is described below (Martin et al., 2007a).

1) Surveillance system components (SSCs), e.g. clinical surveillance or surveillance at abattoir etc. are described by scenario trees. Such components must be able to pick up the hazard in question. Each SSc is a single surveillance activity (defined by the source of data and the methods used for its collection) used to detect one or more hazards in a specified population (Martin et al., 2007a; Hoinville, 2011).

2) The design prevalence is set at national level. This prevalence represents the infection level above which at least one positive animal/herd should be found through the surveillance activities (SSC), with a certain level of confidence (*SSe*).

3) The relative risk between the different population strata is estimated, or obtained from the literature or by expert opinion. For each risk factor represented by a risk category node, a tree branch is given. One reference category is defined by a branch indicating risk equal to 1, while for all other categories (other branches) the relative risk (*RR*) is > 1. At each risk node, the adjusted *RRs* (*ARRj*) are obtained (for the formulas see manuscript IV; Martin et al., 2007a). *ARRs* are then multiplied with the design prevalence, so that the effective probability of infection *(EPIj*) for each branch is calculated. The sensitivity of each component could be calculated as: *EPI* * probability an infected unit (animal or herd) is sampled * *Se* of the test. In some cases, other series of steps that are required to detect a case could be considered. Moreover, not all surveillance components end with a diagnostic test and it is the conditional probability at each step of reaching the next step, that is required (e.g. a series of detection nodes).

4) Usually, the sensitivities of the different surveillance components (CSes) are assumed to be independent from each other and are combined together, to give the overall surveillance system sensitivity (*SSe*). A way of proceeding when CSes are not independent has been proposed by Martin et al. (2007a).

Moreover, the specificity of the surveillance system (*SSp*) is assumed to be 1. Hence, in case one positive sample is found, it is assumed that a further follow up is made in the herd until the presence of infection is either confirmed or rejected (to avoid false positives).

1.9 General approaches of BVD control

Different strategies can be applied to control BVD. The main BVD control measures are: biosecurity (with the goal to prevent introduction of PI animals and dams carrying PI calves in the herd), preventing dams in early pregnancy having contact with infectious animals, removal

of PI animals from the herd as soon as possible and monitoring of the progress of the control measures (Lindberg and Houe, 2005).

Moreover, BVD control programs have previously been classified as systematic or not systematic. In the latter case disease control is made on herd to herd basis, while in the systematic approach, control can be made at regional or country level with coordinated efforts (Lindberg and Houe 2005; Houe et al., 2006).

In Europe, the first systematic control programs were introduced in the ´90s in Denmark (Bitsch and Rønsholt, 1995; Bitsch et al., 2000), Finland (Nuotio et al., 1999), Norway (Valle et al., 2005), Sweden (Alenius et al., 1997; Hult and Lindberg, 2005) and Lower Austria (Rossmanith et al., 2010). Later on they were introduced in Switzerland (Presi et al., 2011), Scotland (www.scotland.gov.uk) Germany (based on vaccination) (Moennig and Greiser-Wilke, 2003; Moennig et al., 2005), and The Netherlands (Mars and Van Maanen, 2005).

The opportunity of setting BVD control programs, has been taken into consideration also in Ireland (Barrett et al., 2011), and France (in Brittany) (Beaudeau et al., 2001a; Beaudeau et al., 2001c; Joly et al., 2005).

In Scandinavian countries, participation in the programs started as voluntary and did not include the use of vaccines. Those programs were mainly based on the control measures mentioned above.

At the beginning of the eradication programs, Finland and Norway had the lowest prevalence of infected dairy herds (around 1 and 9% respectively), while Denmark and Sweden started with around 40% of the dairy herds having PI animals or recent infections (Bitsch and Rønsholt, 1995; Nuotio et al., 1999; Valle et al., 2005; Hult and Lindberg, 2005).

The use of live vaccines in control programs is controversial e.g. due to possible recombination between the vaccine strain and the virus strain that can be present in the field, and due to the lack of marker vaccines (DIVA) (van Oirschot et al., 1999). Furthermore, new discovered strains should be included into the used vaccine, because if this is produced for

strains of only one BVDV species (e.g. BVDV-1), there could be a failure to protect against other BVD strains (e.g. BVDV-2) (Ridpath et al., 1994; Vilček et al., 2001).

1.10 Danish eradication phases and legislation

The Danish BVD eradication program started in 1994 and consisted of four principal steps:

a) Finding herds with increased BVDV antibody titers in bulk milk (bl % > 50 with the Danish blocking ELISA) to identify which herds could harbor PI animals (Houe, 1999).

b) In case a herd was suspected positive, at least 3 young calves older than 6 months of age were tested for antibodies in serum (spot testing). Young animals were targeted, because in the absence of PIs, they should take only few months to become antibody negative after weaning (6-8 months of age) (Houe et al., 1995).

c) In case of confirmed positive BVD status, an antigen ELISA (Rønsholt et al., 1997) was used on antibody negative animals to detect and eliminate PIs from the herd. Herds remained registered as PI for 12 months or more after removal of last born PI animal, i.e. until a control test showed that no PI animals were born. Also BVD free herds, which did not reconfirm their status within a year, were transferred in the group with “undetermined status”, until the free status was established (Bitsch et al., 2000).

d) After removal of all PIs the negative status could be confirmed annually by spot testing (Houe et al., 1995; Houe et al., 2006). After few years this monitoring could be undertaken by testing the BTM for antibodies.

At the beginning of the program, farmers were suggested to test purchased pregnant dams and the born calves, before their introduction into the herd. Since 1996, PI cattle could not graze in common pastures. In 1998, 204 dairy herds and 129 beef herds that were previously classified as free from BVDV were found as newly infected. Then, the legislations were modified in 1999, so that no females over 1 year coming from non-free herds could be moved to the BVDV free herds or common pastures (Bitsch et al., 2000).

Denmark was very proactive in providing information on BVDV status in an open way to all farmers, which probably has made the application of the eradication program more efficient, through the collaboration and the understanding of the farmers.

In 2005, Uttenthal and colleagues analyzed the sequences of BVDV strains collected in Denmark from 36 animals in 1962, 1993, 2002 and 2003. For the latter two years, when the disease was almost eradicated, the identified strains were 1d, 1b and 1e. Those same authors suggested that for early detection of PI calves, PCR could be used instead of antigen ELISA due to the low prevalence of BVDV and to increase the sensitivity for detecting PIs (Uttenthal et al., 2005). As explained above, using the PCR analysis overcomes the problem with the maternally derived antibodies.

The effects of the eradication program during the last years, is shown in figure 1 where the decrease in the prevalence of infected herds between years 2003-2011 is shown.

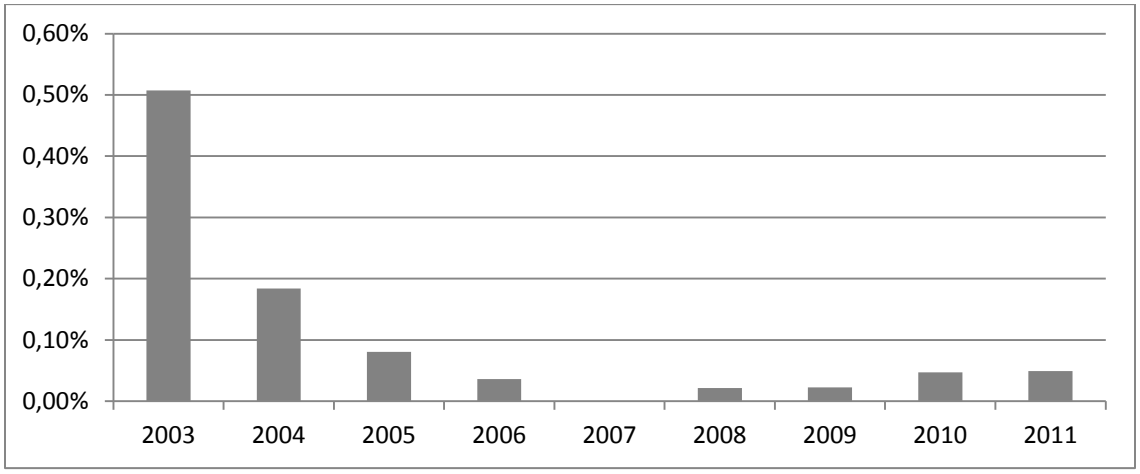


Figure 1. Prevalence of Danish dairy herds with at least one viremic animal in the period between 2003 and 2011. N.B. herd C in manuscript I, was infected in 2006 and persisted until 2010 when the last PI calf was removed. Herd B became infected in 2010 and persisted until 2011.

The cases reported between 2008 and 2011 are due to the three dairy herds (A, B, and C) mentioned in manuscripts I. In all these herds, BVDV-1 strains were isolated. One of the herds (herd C in manuscript I) was supposed to be initially infected in 2006. A wave of PIs was found in 2008. In 2009 and 2010 PI calves were born again because susceptible pregnant heifers were

introduced before the disease clearance procedures were terminated within that herd. In the other two herds PI animals were found in 2010 and 2011 (see manuscripts I and II).

1.11 Current surveillance system

During our study period (2010-2014) dairy herds are screened quarterly by BTM testing with the Danish blocking ELISA, while from beef herds, 4 animals are serum tested every year at slaughter. From beef herds with imported cattle, 2 animals are tested every month at slaughter for a 1 year period.

Though BVDV is a pathogen that affects both dairy and beef herds, in this study we focused on the Danish dairy sector only and we considered beef herds as a separate population in the country. According to the information obtained from the Danish Cattle Federation, Danish dairy herds are very specialized in milk production. Usually, animals (e.g. males) move from dairy to beef herds, and we considered the risk that the infection passed from beef to dairy herds to be low (manuscript III). Hence, we considered beef and dairy herds as 2 distinct BVD-free populations also from a surveillance point of view. Since 2010, no cases have been reported within the beef herds.

In case a dairy herd showed a bl% of 50% and/or bl%>20 in two consecutive bulk milk tests (three months apart), 25-30 animals are tested for antibodies in serum (to find at least one positive with 95% herd sensitivity and assuming a within herd prevalence of 10%). If the infection is confirmed all animals present in the herd are tested for antibodies, while calves which received colostrum and animals antibody negative are tested for BVDV by PCR. During the BVD clearance procedures animal movement is put under restriction till all PIs have been removed from the herd (usually during 1 year period).

1.12 Research needs: Threshold prevalence, high risk period and temporal sensitivity.

In a previous study, Graat et al. (2001) suggested that the main goal of a surveillance system is that "a certified herd that becomes infected is detected timely so that infection of several other certified herds is prevented". Hence, what mainly counts is whether the reproduction ratio R (i.e. the average number of certified herds infected by one "infectious" certified herd) can be kept <1, through the surveillance activities.

The same authors argued that, when a surveillance system is evaluated, some important parameters must be considered. Those are: the sample size (e.g. for a certain herd sensitivity *HSe*), the sampling frequency, the *Se* of the test used, the herd sizes present in the country, the vaccination/immune status of the population and the contacts between herds (as main risk factor for the spread of a pathogen between herds).

Moreover, when an antibody ELISA is used for BTM screening of dairy herds, the prevalence at which the test should give a positive signal (threshold parameter) must be considered. In that case, it is not needed to calculate the sample size to reach a certain *HSe*, since this will be replaced by the *Se* of the ELISA used on the BTM.

Therefore, the sensitivity of a surveillance system based on BTM testing is dependent on the *Se* of the test used and on its threshold prevalence. In fact, it is implicit that the higher the threshold, the higher is the number of animals which must seroconvert before a signal is triggered in the BTM, so that control actions (such movement restriction) can be implemented in the infected herd(s).

The time from a pathogen is introduced into the country, until it is detected by the surveillance activities, can be defined as "high risk period" (*HRP*) (Horst et al., 1997) or "timeliness" (Hoinville et al., 2013). We use the former term, to remark the fact that the longer the time required for detection, the higher is the risk the pathogen is spread from the first case herd to other herds.

Moreover, the probability of detecting a pathogen after a certain time period has been called "temporal sensitivity" (Thurmond, 2003).

In the Danish herd infected in 2010 (herd B in manuscript I and herd A used to validate the model in manuscript II) BVDV was introduced in February, through two PI calves born by two imported Trojan cows. For the other two herds (A and C in manuscript I), the time of BVDV introduction was deduced with more uncertainty, due to the lack of exact information. Because in all three cases the bulk milk antibody titer increased several months after the estimated date of BVDV introduction, concern arose on the temporal sensitivity of the surveillance system based on the use of the Danish blocking ELISA.

Thus, in our studies we first evaluated the temporal sensitivity of the current BVD surveillance system in Danish dairy herds. Then, alternative surveillance strategies aimed at enhancing the temporal *SSe* were investigated. Such alternative surveillance strategies were; a) replacing the Danish blocking ELISA with the SVANOVIR ELISA (Juntti et al., 1987; Niskanen et al., 1989; Niskanen et al., 1991, Niskanen, 1993), and b) proposing a RBS approach where herds at higher risk of introducing BVDV from abroad were tested by individual serum, while other dairy herds were tested in the BTM.

CHAPTER 2

Aims, research questions, materials and methods, answers

2.1 Aim 1, manuscript I

Since 1994, when the Danish BVD eradication program started, the incidence of BVDV infected herds decreased and BVD is now considered an exotic disease in Denmark (Uttenthal et al., 2005). At the same time, the herd size has increased compared to the 90`s, and so the dilution of individual antibodies in larger tanks was expected to have increased as well. The sensitivity of the Danish BVD surveillance system could be affected by those changes, because more antibody positive animals could be needed within infected herds, before a rise in the BTM antibody titer is detected with the ELISA used. Moreover, the sensitivity of the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al.,1997) was evaluated in BTM when the prevalence of infected herds was 26% and herds had in average 42 cows (Bitsch and Rønsholt, 1995; Houe, 1999). At that time, by using the young stock (calves older than 6 months) as Gold standard for defining the true herd infection status, the test showed sensitivity 100% and specificity 62% with cut-off blocking % of 50 (Houe, 1999; Houe et al., 2006). In other Scandinavian countries (e.g. Sweden) the SVANOVIR ELISA®BVDV-Ab (Juntti et al., 1987; Niskanen et al., 1989; Niskanen et al., 1991; Niskanen, 1993) has been used efficiently to eradicate BVD (Alenius et al., 1997; Hult and Lindberg, 2005). Thus we investigated if the latter could perform better than the Danish blocking ELISA, when used on BTM samples, under the current Danish situation.

2.1.1 Research questions

1) What is the current BVD status in Danish dairy herds and the dilution level of individual antibody positive milk in BTM?

2) What is the prevalence of milking cows that must be antibody positive to have detection of antibodies in the BTM?

3) How does the SVANOVIR ELISA perform compared to the Danish blocking ELISA?

2.1.2 Materials and Methods

To answer these questions, first, data collected between 2003 and 2010 was obtained from the Danish Cattle Federation and were analyzed using the freeware R (version 2.13.2, R Development Core Team, 2010). The dataset included the registration number of the Danish dairy herds (CHR number), information on the amount of milk delivered per herd, BTM antibody values, number of cows per herd, and number of viremic animals eventually present in the herd. The milk produced daily per cow was estimated, according to the daily milk delivery per herd and the percentage of cows present in the milking group. Based on our knowledge of the Danish dairy industry we assumed that between 12 and 20% (usually 17%) of the cows present in a herd are dry and do not contribute to the BTM.

Secondly, the Danish blocking ELISA and the SVANOVIR ELISA were compared in the laboratories of Lindholm by testing (i) positive individual diluted milk and sera (from herd A in manuscript I), (ii) artificial pools of milk and (iii) BTM samples collected from herds B and C (for further details see manuscript I).

The diluted milk samples were used to estimate the minimum prevalence of seroconverted milking animals needed, to detect antibodies in the BTM with the ELISA used. The artificial milk pools were tested to estimate the relation between values in the ELISA and the concentration of positive milk present in the pool. Finally, the BTMs of herds B and C were tested in different periods, to investigate the time required for the BTM to become negative again after removal of the last born PI calf.

2.1.3 Answers

We found that, the annual amount of milk produced in the country changed slightly (from 4.4 to 4.7 billion kg) during the investigated years (2003 vs. 2010), while the median herd size, the herd´s production (Figure 2) and the daily milk yield per cow increased steadily (Table 1 in manuscript I).

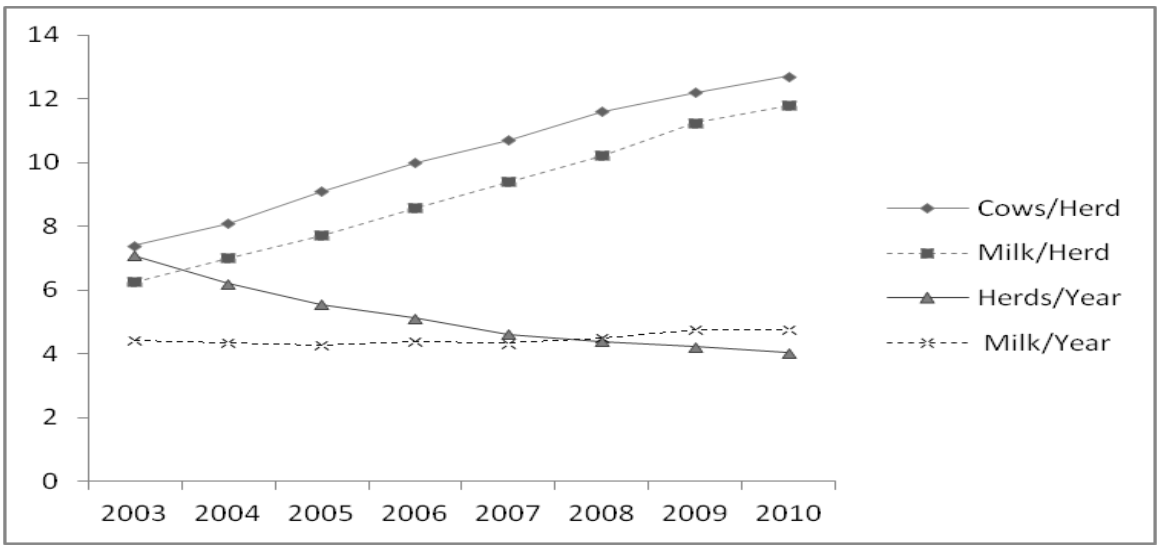


Figure 2 (Fig. 1 in manuscript I). Changes in herd size and milk production from 2003 to 2010. Cows/Herd = median herd size (divided by 10); Milk/Herd = milk produced per herd (in 100,000 kg). Herds/Year = number of Danish dairy herds (in 1,000), which delivered milk from January to December; Milk/Year = national milk production (in billion kg of milk).

Consequently we proved that milk from a single seroconverted animal could be more diluted within the BTM compared to the past (see Table 1 in manuscript 1). Moreover, the prevalence of Danish dairy herds with viremic cattle decreased from 0.51% (in 2003) to 0.02% (in 2010). Accordingly, antibodies reached undetectable levels in the BTM, and in 2010, 75% of BTM samples had a bl% of 0, while the remaining 25% had a median bl% of 5 (3rd quartile = 9%). The maximum value was bl% = 80 in the herd infected in 2010 (herd B in manuscript I). Those findings suggest that Danish cattle can be considered naïve to BVDV.

The Danish blocking ELISA appeared capable of detecting an increase in the bulk milk antibody titer (bl%>0) when at least 50% of the milking cows are positive, while the SVANOVIR®BVDV-Ab could detect a medium level of antibodies (PP > 2) when 1/128 milking cows is positive in the herd (0.78%)(manuscript I, Fig. 3).

Moreover, in the experiments with the artificial pools of milk, we showed that values in the SVANOVIR ELISA (PP) better relate to low concentrations of antibody positive milk in a BTM sample, than values in the blocking ELISA (bl%). In individual serum, the two ELISAs performed equally well (manuscript I, Fig. 4).

Using BTM samples from herds B and C, we found that if the SVANOVIR ELISA is used, the time needed for the BTM to be classified as BVDV free again after removal of the last born PI calf is longer than when the blocking ELISA is used (manuscript I, Fig. 2). Nevertheless, it could be sufficient that a low antibody level is shown in the BTM, for the herd to be classified as free from BVDV (e.g. class 0 or 1 in the Swedish system) (Alenius et al., 1997).

2.2. Aim 2, manuscript II

In study I, we showed that a higher threshold prevalence is required for the Danish blocking ELISA than for the SVANOVIR®BVDV-Ab, to trigger a positive signal in the BTM. This suggested that the time needed to find newly infected herds (e.g. after import of infected cattle) by BTM testing, could be longer if the blocking ELISA is used. Hence, the main aim of study II was to investigate if the detection time of the two ELISAs was significantly different.

2.2.1 Research questions

1) Using the Danish blocking ELISA, how long would it take to detect antibodies in the BTM following BVDV introduction into the herd?

2) Using the SVANOVIR ELISA or the BVD/MD p80 Insitut Pourquier ELISA, would the detection time change significantly compared to the Danish blocking ELISA?

3) Would the herd size affect significantly the detection time?

2.2.2 Materials and Methods

In this study, a stochastic model was developed in R, in order to simulate BVDV spread within a typical Danish dairy herd. Thereafter, we used this model to estimate the BVD detection time of three antibody ELISAs: a) the Danish blocking ELISA, b) the SVANOVIR®BVDV-Ab, and c) the indirect ELISA BVD/MD p80 Institut Pourquier. The latter is used in France where BVD is endemic (Beaudeau et al, 2001a). The thresholds prevalence used were 50%, 6%, and 9%, respectively, based on previous studies (manuscript I, Niskanen, 1993; Beaudeau et al, 2001a). The impact of the herd size and the effect of introducing a PI calf or a TI milking cow on the detection time, were also evaluated.

The validation of the model was mainly carried out by comparing the predicted incidence of PI calves and the predicted detection time, with records of an infected Danish dairy herd (Herd

B in manuscript I, Herd A in manuscript II). That herd became infected with BVDV in February 2010, but the BTM was classified positive (with the blocking ELISA) only in November (bl% 65). At that time, two PI calves (which were born from imported cows) were still alive and were kept in another stable, since one month from birth. The calves stable was situated 200 meters away from the milking group. Thus, the day of BVDV introduction in this herd could be traced back with high confidence.

To estimate the detection time of each ELISA, simulations were made with different herd sizes (small = 70, medium = 150 and large = 320 cows) and introducing one PI calf or one TI milking animal in the simulated herd. When the threshold of antibody positive milking cows was reached, the model output was printed as: cumulative overall number of born PIs and number of days elapsed between BVDV introduction and detection of antibodies in BTM.

2.2.3 Answers

The model can give an insight into patterns of births of PI calves (Figure 3), virus spreading and immunization within the milking group (Figure 4 and 5). Predictions from the model fitted the data from the infected herd. The median number of born PIs predicted by the model was 27 (5^{th} percentile 7; 95^{th} percentile 54), while the predicted detection time was 301 days (226; 564). According to the data from the Danish Cattle Federation, 29 PIs were born in the herd and detection occurred at 287 days form BVDV introduction. Moreover, the predicted weekly prevalence of live PI calves (Fig. 6) matched the within herd prevalence (1-2%) of PIs that is usually reported in literature (Houe, 1999). Additionally, it can be noted that after week 41 (when the BTM was found positive), such a prevalence reduced to 0% because we simulated the fact that after detection, all calves were moved far away from the milking group by the farmer (until results of serum testing were received) (Fig. 6). For the same reason virus spread stopped within the milking group (Fig. 4).

Regarding the detection time of the three tests, we found that the SVANOVIR®BVDV-Ab is significantly quicker than the other two ELISAs to detect newly infected herds. Moreover, the detection time increased significantly with the considered herd sizes (Table 4, manuscript II).

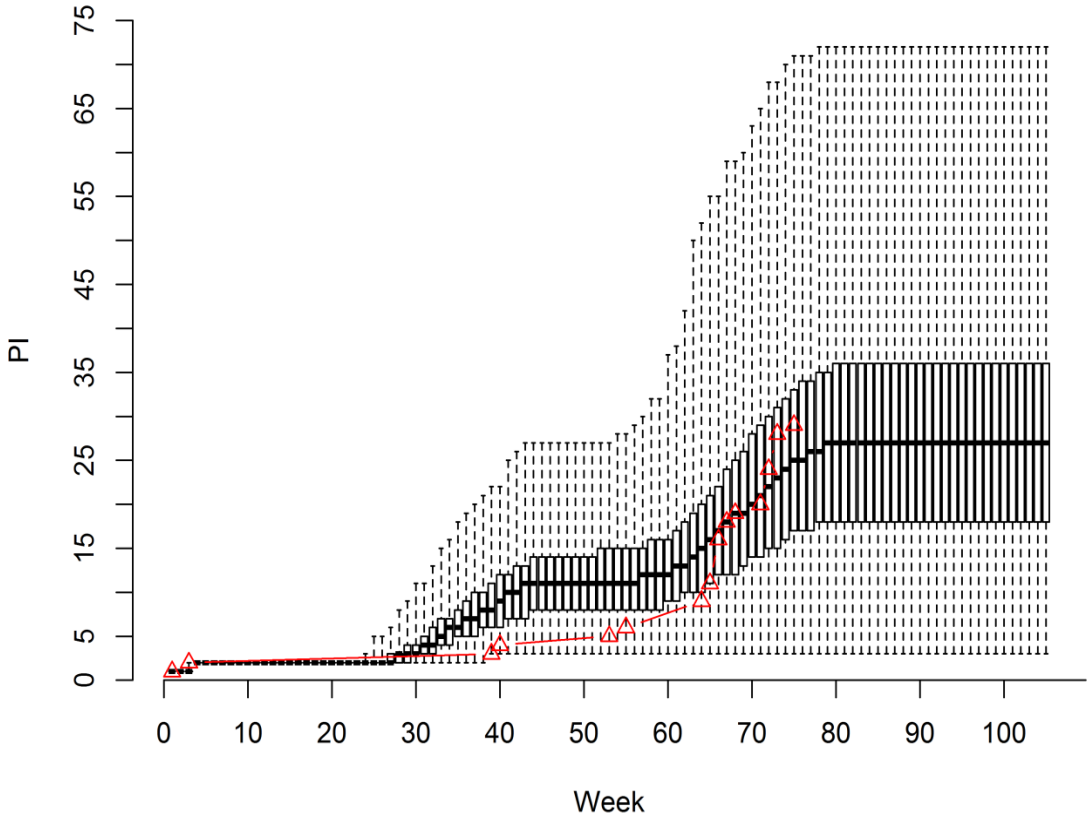


Figure 3 (Fig. 1 in manuscript II). The predicted cumulative number of born PIs (per week) in herd A, shown in a box-plot (black line = median; bars = 1st and 3rd quartiles, dashed lines = minimum and maximum). The red line represents observed data. The red triangles represent the weekly cumulative number of born PIs.

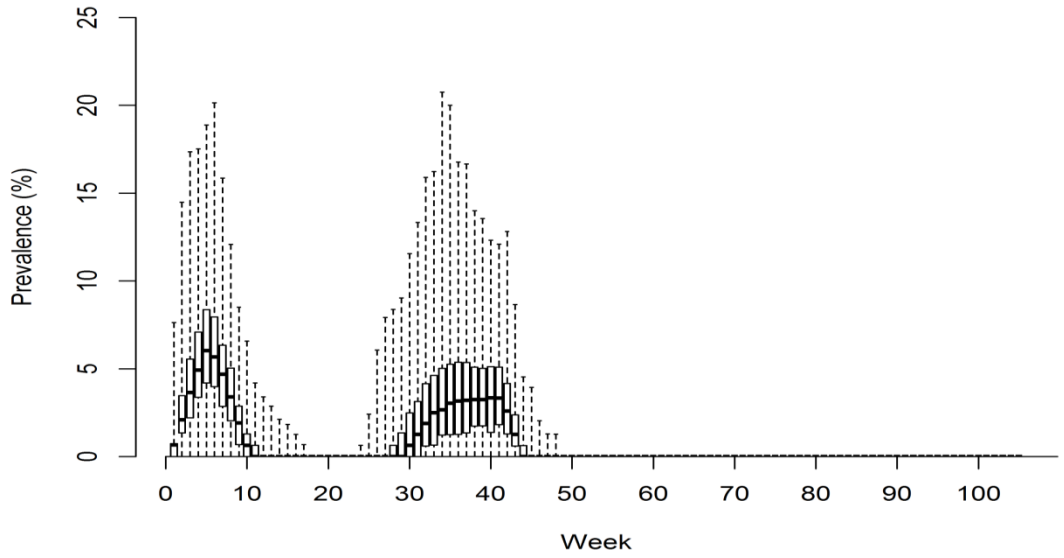


Figure 4 (Fig. 2 in manuscript II). Box-plots representing the predicted weekly prevalence of viremic (TI) milking cows. The first peak between weeks 1 and 11 is caused by the two PIs we introduced mechanistically at days 1 and 21. The second peak (weeks 29-44) is caused by the new born PIs.

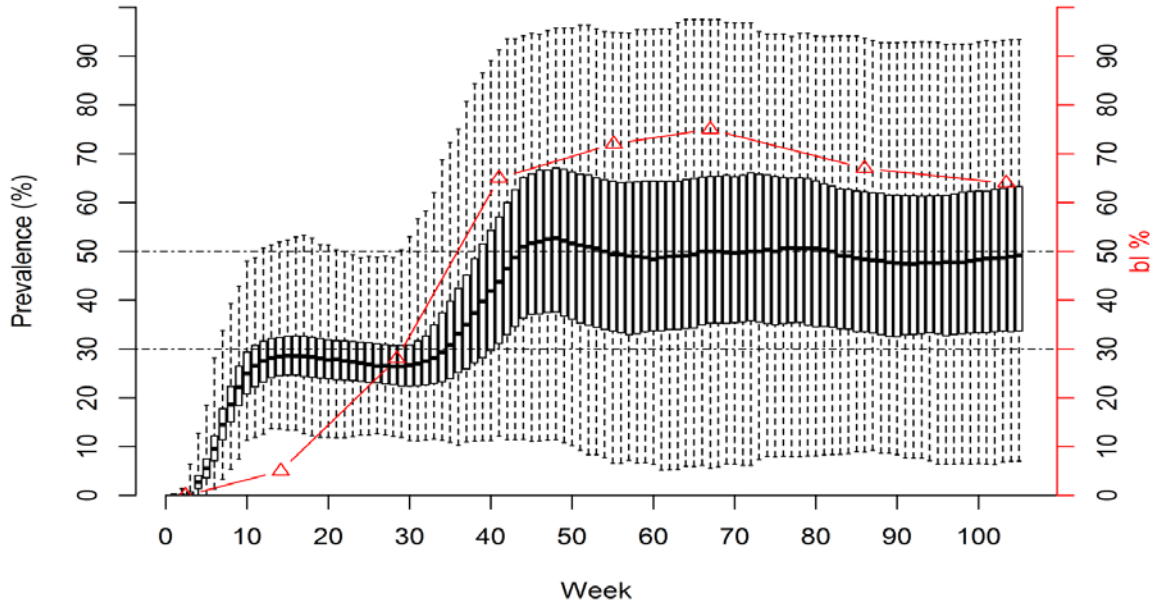


Figure 5 (Fig. 3 in manuscript II). Box-plots representing the predicted weekly prevalence of antibody positive milking cows in the herd (black line) and the BTM values (red triangles) registered in the database of the Danish Cattle Federation (values in blocking % according to the Danish blocking ELISA, see right axes). The horizontal dashed lines represent the threshold prevalence (30 and 50%), at which the BTM was expected to be classified as positive (bl% > 50 and/or two consecutive samples >20%).

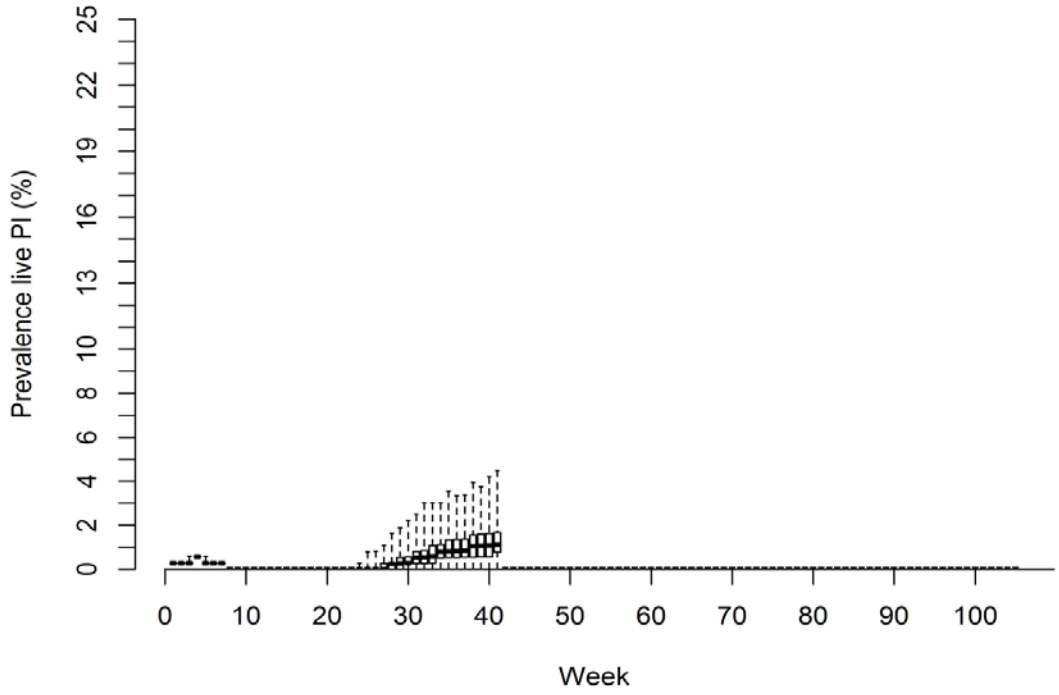


Figure 6. Box-plots representing the predicted weekly prevalence of live PIs before detection by BTM testing. The first peak between weeks 1 and 11 is caused by the two PIs we introduced mechanistically at days 1 and 21. The second peak (weeks 29-44) is caused by the new born PIs. N.b. This figure was not used in manuscript II, because data on cumulative born PIs per week was used instead (Fig. 3 above).

2.3. Aim 3, manuscript III

In countries where BVD is eradicated, BVDV could be introduced from abroad, e.g. due to import of infected live animals. Hence, estimating the risk of BVDV introduction and the impact of risk mitigation strategies is fundamental, to prioritize measures of biosecurity/surveillance, and in order to maintain the BVD free status. This also gives the advantage of exporting BVDV free animals to other countries. Thus, the aim of study III was to quantitatively assess the likelihood of BVDV introduction into at least one Danish dairy herd.

2.3.1 Research questions

1) What is the risk that BVDV is introduced into Danish dairy herds from abroad?

2) What introduction pathways represent the highest risk?

3) What are the risk mitigation measures to prioritize, in order to reduce the risk to an acceptable level?

4) For which risk factors is there a lack of knowledge and higher uncertainty?

2.3.2 Materials and Methods

The risk of BVDV introduction was estimated per year and per trimester. Moreover, the impact of risk mitigation measures such as compulsory testing for all live animals imported and disinfection of tools used for hoof trimming, were investigated.

Data (2010) on import of live animals, semen and embryos, use of vaccines and on truck visits was obtained from the Danish Cattle Federation and analyzed. Information on veterinarians and hoof trimmers practicing in Denmark and abroad was obtained by contacting several institutions and by expert opinion. The opinion of experts was gained by questionnaires.

For instance, to estimate the probability that BVDV is not removed by the truck disinfection procedures, we contacted 3 virologists and 3 epidemiologists from Denmark and from other European countries (Germany, The Netherlands and Sweden). We asked the experts to give a minimum, a maximum and a most likely probability estimate. Thereafter, we combined the opinion of the experts in a Pert distribution, where the minimum and the maximum were represented by the median of the minimum and maximum estimates of the six experts, while the mode was represented by the median of their most likely estimates (see distributions used for expert opinions in Table 7-8 of manuscript III)

Results of the data analysis and inputs from the experts were fed into five stochastic scenario trees developed in @RISK 6 (*PAnim, PSem, PEmb, PTruck* and *PTrim,* in manuscript III). Each scenario tree represented a possible BVDV introduction path in Danish dairy herds. Hence, the risk of BVDV introduction was estimated per introduction pathway (live animals, semen, embryos, trucks contaminated abroad and hoof trimmers practicing abroad). Then, the final overall risk was estimated by combining outputs of the five scenario trees altogether as:

$$1-[(1- PAnim)*(1- PSem)*(1- PEmb)*(1- PTruck)* (1-PTrim)] \quad \text{(Eq.1)}$$

Scenario trees for veterinarians, cattle shows and vaccines were not made, because according to the institutions and experts we contacted, we deduced that they would not represent a relevant risk.

2.3.3 Answers

We estimated that the major sources of infection in the country are the imported live animals, especially if they arrive from endemic countries and when testing of blood is not compulsory in Denmark. The second most important source was represented by hoof trimmers crossing borders and not always disinfecting the tools. The risk of infection for the other three introduction pathways (semen, embryos and trucks) was rather low (manuscript III). Without new mitigation measures, at least one introduction per 9 years could be expected.

By making the testing of all imported animals compulsory and disinfecting the tools used for hoof trimming, the risk could be reduced to 1 BVDV introduction per 33 years.

2.4 Aim 4, manuscript IV

The final study of the Ph.D. project aimed to (a) evaluate the temporal sensitivity of the Danish BVD surveillance system (*SSe*) by using the information from the first three studies (manuscripts I, II and III), and (b) to suggest how to optimize[1] the *SSe* (e.g. using alternative testing strategies). We also investigated the possibility of testing herds at higher probability of BVDV introduction (importing live cattle) using individual serum (*ImpoCattle* herds), while in other herds (*NoImpoCattle*), the BTM testing was maintained. This categorization was made according to study III, where we found that, under the current situation (without additional risk mitigation measures) the most important source of BVDV introduction into Danish dairy herds is the import of live animals.

The confidence that the herd prevalence was below the assumed between herds design prevalence (P_H), was also estimated based on the calculated temporal *SSe.*

2.4.1 Research questions

1) Considering outputs of studies I, II and III, what is the temporal SSe of the current BVD surveillance system?

2) Would using the SVANOVIR ELISA improve the SSe?

3) Would testing individual serum in herds at higher risk of infection improve the SSe (risk based approach)?

4) According to the estimated temporal SSe, what is the confidence in complete freedom (PFree) from BVDV (P_H < 1 infected dairy herd) and the confidence in low (PLow) herd prevalence (P_H <0.2%)?

[1] In this thesis the term ”optimization”is meant the increase in the temporal *SSe*.

2.4.2 Materials and Methods

The temporal *SSe* of the surveillance system was estimated according to the ELISA used, herd structure, risk of BVDV introduction per herd category and time elapsed from BVDV introduction to day of herds testing (High Risk Period or *HRP*). Four surveillance strategies were investigated:

a) Testing all Danish dairy herds in BTM with the blocking ELISA (current surveillance system).
b) Testing all Danish dairy herds in BTM with the SVANOVIR ELISA.
c) Using the blocking ELISA, or d) the SVANOVIR ELISA to test BTM in *NoImpoCattle* herds and to test individual serum in *Impocattle* herds. Hence in strategies "c" and "d", two surveillance components were simulated (based on BTM and serum testing, respectively).

Two BVD infection scenarios were considered for Denmark:

1) The Danish dairy flock is free from infection and BVDV is introduced from abroad into 1/4109 herds (P_H 0.02%);

2) BVDV is introduced in few Danish dairy herds (P_H 0.2%, corresponding to 8/4109 infected herds). In that case, the between herds design prevalence is the same set up by the World Animal Health organization (OIE) to substantiate official free status from enzootic bovine leucosis (EBL) (OIE, 2010 art. 11.9.2) infectious bovine rhinotracheitis (IBR) (OIE, 2010 art 11.11.2) and bovine tuberculosis (bTB) (OIE, 2013 art. 11.6.2).

In both cases (1 and 2), we considered herd infections due to introduction of one PI calf or one TI milking cow, and *HRP* of 90 or 365 days. Those two time intervals, lead to the temporal *SSe,* by testing quarterly and testing on yearly basis (after BVDV introduction to the country), respectively.

Stochastic scenario trees (see Figures, 7, 8 and 9, below from manuscript IV) were used to estimate the temporal *SSe*. The related confidence (*PFree*) in complete freedom from BVDV (P_H <0.02%) and the confidence (*PLow*) in low herd prevalence (P_H <0.2%) corresponded to the negative predictive value (NPV) of the surveillance system (Martin et al., 2007a). Hence, *PFree*

and *PLow* represented the confidence that a country classified as "negative" to BVD by the surveillance system is truly negative. Or, in other words, *PFree* and *PLow* represented the confidence that the prevalence of BVD infected dairy herds was truly below the assumed design prevalence (P_H), if no positive herds are detected by the surveillance system.

The Danish dairy herds delivering milk in the last trimester of 2010 (4109 herds in October) were divided into the two main risk categories: *ImpoCattle* (8 herds) and *NoImpoCattle* (4101 herds). Thus, by definition, all imported cattle went to the *ImpoCattle* category. Then for each herd category we estimated the annual number of (i) imported semen doses, (ii) imported embryos, (iii) truck visits and (iiii) the visits by hoof trimmers. Thereafter, information from steps "i" to "iiii" was fed into the stochastic scenario trees developed in manuscript III, to estimate the annual risk of BVDV introduction in each risk category.

For the BTM testing, the within herd design prevalence P_U from Martin et al. (2007a) corresponded to the threshold prevalence of antibody positive milking cows, at which the BTM was classified positive by the ELISA used, with the assumed test sensitivity (*Se*). Thus, the threshold was set to 6% for the SVANOVIR (Niskanen, 1993) and 50% for the blocking ELISA (manuscript I). The *Se* ranged from 93.4% to 99.6% for the SVANOVIR (Lindberg, 2000), while it was 100% for the blocking ELISA (Houe, 1999). Instead, when we considered the testing of individual serum in herds at higher risk of BVDV introduction (*ImpoCattle* category), we replaced the *Se* (of both tests) with herd sensitivity (*HSe*) 95%. This is the herd sensitivity used by the Danish Cattle Federation to detect at least one antibody positive animal in a herd classified as BTM positive, assuming a 10% within herd prevalence. Hence, in that case, P_U was the overall within herd prevalence of antibody positives (considering cattle which are milking and cattle located in other groups).

The probability (*PTR*) that the threshold prevalence (or P_U) was reached at the day of herd testing was estimated using the simulation model developed in study II. We knew from that study, that the herd size affects both the detection time and the *PTR*. Thus, we estimated the *PTR* for the minimum, maximum and most common herd size in each category, after introduction of a PI calf or a TI milking cow, and according to the assumed *HRP* (90 or 365 days).

In the *ImpoCattle* category, herds had size between 24 and 1070 cows (median 180), while in the *NoImpoCattle* category the size ranged between 1 and 1185 cows (most of the herds were assumed to have around 150 cows). Thereafter, the *PTR* values from Table 3 and 4, in manuscript IV, were set with a Pert distribution in the stochastic scenario trees that we used to estimate the temporal *SSe* (Fig. 7, 8, and 9 below). The node ("Threshold reached ?") used for the *PTR* values was therefore a detection node, together with the node ("ELISA") used for the *Se* of the test (Martin et al., 2007a).

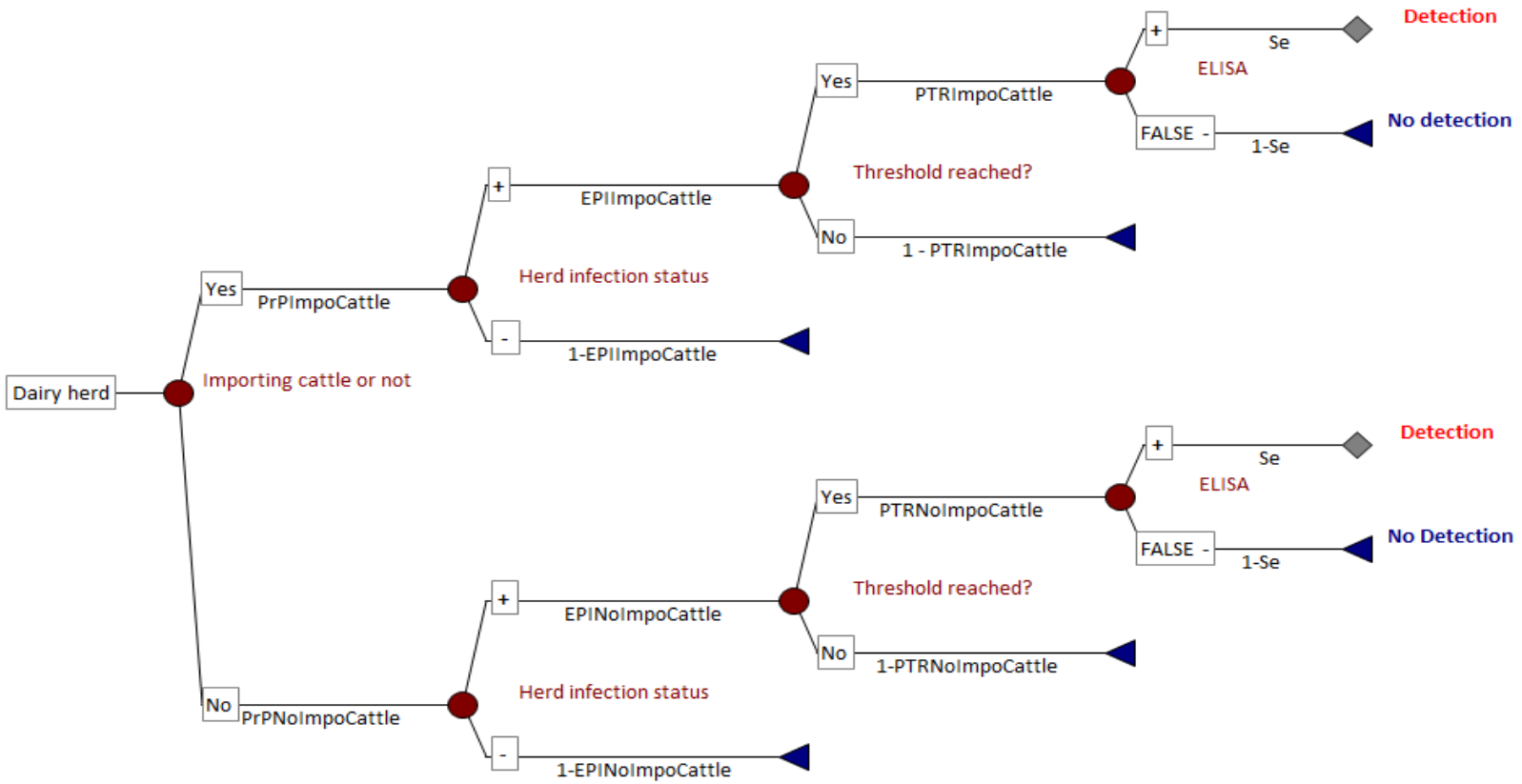


Figure 7 (Fig. 1 in manuscript IV). Stochastic scenario tree for the comprehensive surveillance component where all Danish dairy herds are tested in BTM (strategy "a" and "b"). $PrP_{ImpoCattle}$ and $PrP_{NoImpoCattle}$ = proportion of dairy herds within the *ImpoCattle* and *NoImpoCattle* category. $EPI_{ImpoCattle}$ and $EPI_{NoImpoCattle}$ = effective probability of infection within the *ImpoCattle* and *NoImpoCattle* category. $PTR_{ImpoCattle}$ and $PTR_{NoImpoCattle}$ = probability that the threshold prevalence is reached within the milking paddock at 90 or 365 days from BVDV introduction within herd(s) of the *ImpoCattle* and *NoImpoCattle* category (Pert distributions based on Table 2, manuscript IV). *Se* = sensitivity of the antibody ELISA used (Danish blocking ELISA or SVANOVIR) on BTM, when the threshold prevalence of seroconverted milking cows is reached.

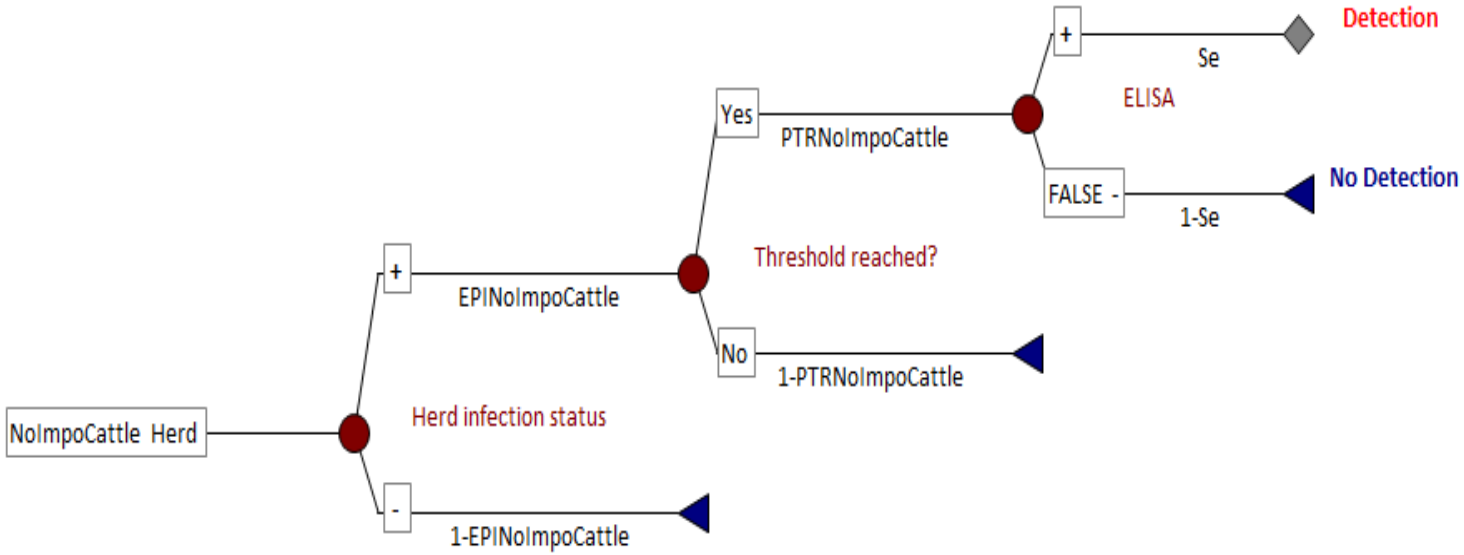


Figure 8 (Fig. 2 in Manuscript IV). Stochastic scenario tree for the surveillance component of *NoImpoCattle* herds tested on BTM samples (surveillance strategy "c" and "d"). Legend as in Fig. 1. In that case, the node "Importing cattle or not" is not needed, since in this tree we only consider herds which did not import live animals.

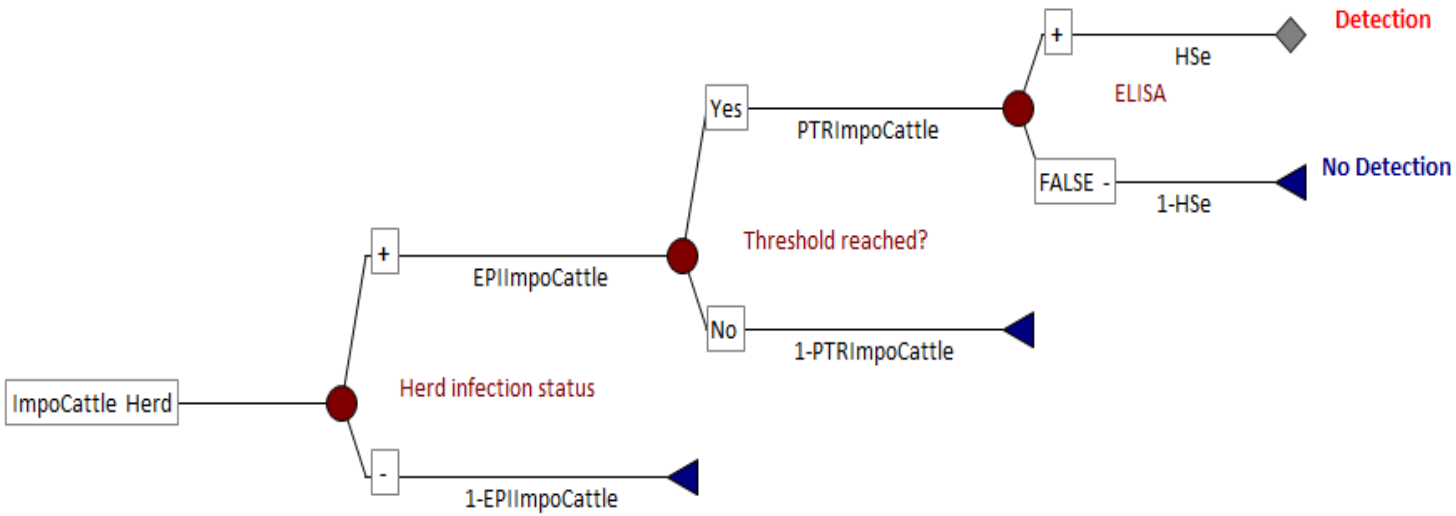


Figure 9 (Fig. 3 in Manuscript IV). Stochastic scenario tree for the surveillance component of *ImpoCattle* herds tested on individual serum samples (in surveillance strategy "c" and "d"). $EPI_{ImpoCattle}$ = effective probability of infection in the category. $PTR_{ImpoCattle}$ = probability that the threshold prevalence (10%) is reached within the overall herd, at 90 or 365 days from BVDV introduction (Pert distribution based on Table 3 of manuscript IV). *HSe* = herd sensitivity to find at least one seroconverted animal at the within herd prevalence 10%. The *HSe* was assumed to be the same for the Danish blocking ELISA and the SVANOVIR ELISA. Hence we assumed that enough animals within a herd would be tested in serum, to reach *HSe* with the ELISA used.

2.4.3 Answers

The two tests gave similar high *SSe* and *PLow* estimates (> 95%) only when we considered BTM testing in all Danish dairy herds at 365 days from introduction of a PI calf in at least 8 dairy herds.

In the other investigated scenarios, the temporal *SSe*, the *PLow* and the *PFree* were higher with an *HRP* of 365 days, than with an *HRP* of 90 days. Estimates were usually higher for the SVANOVIR than for the blocking ELISA, and when a PI calf rather than a TI cow was introduced to the herd(s) (see Table, 4 in manuscript IV).

For instance, with the Danish blocking ELISA, the median temporal *SSe* was around 65% if a PI calf was introduced into at least eight dairy herds and all Danish dairy herds were tested in BTM after 90 days (current system, or surveillance strategy a). The related *PLow* was 72%. When a PI calf was introduced into one herd and the same testing strategy was used, the temporal *SSe* was 12%, while the related *PFree* was 52%. With the SVANOVIR (strategy b) these estimates were around 99%, 99%, 42% and 62%, respectively (Table 4, manuscript IV).

Hence, the temporal *SSe* and the related *PFree/PLow* could be improved remarkably, if the blocking ELISA was replaced by the SVANOVIR to test BTM in all Danish dairy herds. Testing *ImpoCattle* herds in individual serum and *NoImpoCattle* herds in BTM (strategies c and d) would not increase the temporal *SSe* noticeably (independently of the ELISA used), due to the low number of dairy herds importing cattle.

CHAPTER 3

Discussion

3.1. Considering BTM testing from a new point of view

Antibody ELISAs are considered the tests of preference to be used on BTM, because they combine sensitivity and cost efficiency of the surveillance system. It is also known that the concentration of BVDV antibodies in the BTM relates well to the prevalence of serum positive milking cows present in the herd (Niskanen, 1993). Therefore, antibody ELISAs are used for the regular analysis of BTM samples, to monitor and validate the BVDV-free status in dairy herds (Niskanen et al., 1991; Niskanen, 1993). The main advantage of testing the BTM is that, in each herd, only one easily available sample is analyzed instead of sampling from all individuals present.

On the other hand, the accuracy of the ELISA used can vary with the time elapsed from the infection day of the herd, as it has been shown in manuscript II. Especially in recently infected large herds, false negative BTM samples could be found when only few animals have sero-converted, and a long time could be needed before finding an increased antibody titer in the BTM (see figure 3 in manuscript II). For instance, in the case of large herds (≥ 320 cows), detection could take more than a year (Table 4, in manuscript II). Usually, veterinary authorities assume that this problem can be overcome by repeated analysis of the BTM (e.g. with 3-4 month intervals) and/or using a more sensitive ELISA.

In our opinion, the BTM could be considered an analogue to the single animal (until this seroconverts), where the BVD infection process shows three main phases: 1) the susceptible animal (**S**) is exposed to the virus, but is not viremic neither is immune (incubation phase, 4-7 days), 2) the animal becomes viremic and starts shedding the virus, but has not antibodies (infectious status **I**, 10-14 days) and 3) the animal recovers, it stops shedding the virus and becomes immune lifelong (recovered status, **R**). Thus, if the antibody ELISA is used to test the immune status of an animal, at least 2-3 weeks should elapse between the day the animal was exposed to the virus and the day it seroconverts.

These phases are partly valid also at the herd (BTM) level (until antibodies are detected), though the system is far more complex, because more animals are involved and the

performance of the test depends on the milk tank composition (e.g. this has been shown for *Neospora caninum* by Frössling et al., 2006).

In phase 1, the BVDV is introduced into the herd (e.g. by a PI calf). At this stage, in naïve Danish dairy herds, the BTM would be free of antibodies, unless some very old animals which were infected in the past (e.g. 10 years ago before eradication) are present in the herd, and/or some antibody positive cow is imported from abroad (e.g. cows which carry PI calves or Trojan cows). In phase 2, the virus is spread to the farm mates. At this stage, the BTM could still be free of antibodies (or antibodies are present at very low level). In phase 3, a sufficient number of milking cows that were infected seroconverts and shed the antibodies in the BTM. Following phase 3, when the threshold prevalence is reached within the milking group, the herd will be classified as BVD positive by BTM testing with the antibody ELISA.

If the infection status is confirmed by individual blood testing, the BVDV clearance procedures are carried out to remove all PIs from the herd. Thereafter, differently from the individual animals, which remain immune lifelong (status **R**), the herd will become naïve again when all old immune cows are replaced by new naïve animals born after removal of PIs. This phase could require some years, and as we showed for herds B and C in manuscript I, it could take longer with the SVANOVIR than with the blocking ELISA. During this phase, eventual new BVDV introductions to the herd can be monitored by testing young animals (older than 6 months), which were born after removal of PIs (Houe et al., 1995).

Moreover, each of the phases described above, will be affected by 1) the type and the number of the introduced infected animals (e.g. PI, and/or TI, and/or a dam carrying a PI fetus), 2) the number of susceptible and milking animals present in the herd, 3) the ELISA used to detect the antibodies in the BTM, and 4) the time elapsed from the day of BVDV introduction to the day of BTM testing (*HRP*).

We tried to investigate the combination of these factors and we created a flexible stochastic simulation model (manuscript II), which can be easily adapted for different herd structures and diseases. Information on disease epidemiology, herd size, and farmer´s practices (e.g. elimination of new born male calves) can be combined in the model. Thus, the model was used

to predict how the infection and immunization processes develop within the milking paddock, according to the incidence of PIs in the herd (see figures from 3 to 6 above). The individual milk yield and the antibody titer of the cows could also play a role, but we used a simplification (see Chapter 5).

Only few BVD stochastic models have been validated using data on PI calves born in the herd (Sørensen et al., 1995; Viet et al., 2004). Our model was validated using data from a recently infected dairy herd, where the date of BVDV introduction could be traced back with high confidence. Although in reality other infectious sources could have played a role (e.g. imported contaminated semen and/or embryos, truck visits, and visits by hoof trimmers), we believe that the two PI calves born in the herd in February 2010 were the most likely cause of the BVDV introduction in such a farm. In fact, as we showed in manuscript III, imports of live animals represent a higher risk of BVDV introduction compared to the other infection means we considered.

Additionally, the threshold prevalence of antibody positive milking cows, that was needed to reach the antibody detection limit by the ELISA used in the BTM, was introduced into the model.

For the SVANOVIR ELISA this threshold was estimated by Niskanen (1993). This author suggested that in herds where 6% of the cows are antibody positive (milk titer 1:16), the test should give a positive signal. In the case of the Danish blocking ELISA, the threshold prevalence needed to be estimated. For that reason, we decided to make the experiments in study I, and to compare the two ELISAs. Then, for the SVANOVIR ELISA (in manuscript II) we decided to use the threshold given by Niskanen (6%) because this gave a more conservative detection time. In fact, in study I, it appeared that the SVANOVIR could start giving a positive signal in the BTM with 1 cow positive out of 128 (threshold prevalence = 0.78%). The mean milk PP value, between cows tested in diluted milk, was always above the cut-off 2 that represented a low level of antibodies in the BTM (see Fig. 3 in manuscript I). Those animals had individual milk PP that ranged between 9 and 19.

Thereafter, we compared the antibody ELISAs on their detection time. For the BVD/MD p80 Institute Pourquier ELISA, the threshold was found in the literature. According to Beaudeau et al. (2001a), herds with less than 9% of the milking cows positive should test negative in the BTM. Therefore, we set the threshold at 9%.

The findings of study II showed that, for all the three ELISAs, the detection time can be significantly affected by the herd size and the threshold prevalence is an important parameter to consider. This is important for BVD surveillance, and together with previous studies that were carried out for other cattle diseases (Graat et al., 2001; Frössling et al., 2006), paves the road for considering the sensitivity of antibody ELISAs used in BTM testing from another perspective than is currently the case. In fact, usually, the sensitivity of tests used on BTM is reported without the threshold prevalence for which such "herd sensitivity" is valid. We believe that when the sensitivity is estimated, the threshold prevalence should be reported as well.

3.2 Risk of introduction of BVDV from abroad and risk mitigation measures to prioritize

To keep the advantage of having BVDV free herds, it is fundamental to reduce the risk of BVDV introduction to a low level. To achieve this goal we assessed quantitatively the likelihood of BVDV introduction from other countries to at least one Danish dairy herd. Then, we investigated measures of risk mitigation.

For these reasons we first studied what would be the main sources of BVD introduction from abroad, taking into account the opinion of the stakeholders of the Danish dairy industry. Therefore, we received datasets from the Danish Cattle Federation on the variables we prioritized for the risk assessment. The main guidelines of international institutions as the OIE were followed.

According to the estimates we obtained, it seems convenient to make compulsory the testing of all individual imported animals, because only few herds and animals are involved. Advising hoof trimmers on the importance of disinfecting the tools used abroad is also important.

Moreover, the model we made was able to reflect the changes in the risk within a year period (see figure 2 in manuscript III). In case new introductions will be found in the future, the model can be further validated as we did for 2010. In fact, the model reflected the risk according to the quantity of imported animals, semen etc. and their country (endemic vs. free) of origin.

Additionally, in our risk assessment, we considered the possibility of introducing BVDV through the import of TI animals, which are usually considered of low importance compared to PIs. We think that it is preferable to consider also TIs, since as stated by others, acutely infected cattle may be the primary source of virus introduction into naïve herds and maybe responsible for continued circulation of BVDV within large herds (Moerman et al., 1993, Brock, 2003). For that reason, we considered a successful BVDV introduction to Danish dairy herds, each time a transient infection was caused and/or a viremic animal (TI or PI) was imported.

3.3. Evaluation and optimization of the Danish BVD surveillance system

The final step of the Ph.D. project was to evaluate the performance of the current Danish BVD surveillance system, taking the time from BVDV introduction (to the country) into account. Furthermore, possible alternative surveillance strategies were investigated, to suggest how to optimize the system. Therefore, in this thesis, the term “optimization” meant the maximization of the *SSe* with its related confidence in freedom (*PFree*) from BVDV and the confidence in low herd prevalence (*PLow*).

Usually, to reach the predefined target of confidence in pathogen/disease detection (*SSe*) and freedom, two main options are available: a) sampling more units (but in Denmark all dairy herds are already tested) and/or b) using a RBS approach with more sensitive testing in high risk strata (e.g. taking relatively more samples than in low risk strata). Whether this is fruitful or not, will depend on the magnitude of the relative risk (*RR*) as well as the number of samples taken in each high risk strata.

We considered the opportunity of having two instead of one surveillance component. Hence as a RBS approach, we considered testing individual serum in herds importing cattle and testing the BTM in other herds, by using the Danish blocking ELISA or the SVANOVIR (surveillance strategies "c" and "d" in manuscript IV). By doing so, we expected to have higher temporal sensitivity in *ImpoCattle* herds, because the within herd design prevalence used was 10% (considering the whole herd) rather than using threshold prevalence 50% (within the milking group). For that reason we used two stochastic scenario trees, one for each surveillance component (Figures 8 and 9 above). We found that in *ImpoCattle* herds the PTR values were higher using individual serum testing than when BTM was tested (Tables 2 and 3, manuscript IV). Nevertheless, the overall temporal *SSe* was not remarkably enhanced in testing strategies "c" and "d", because in 2010 few herds imported few cattle and the *RR* of BVDV introduction into the *ImpoCattle* category was slightly higher than that used in the reference risk category (*RR*=1 in the *NoImpocattle* category) (see manuscript IV). Therefore, maintaining BTM surveillance in all Danish dairy herds seems the most efficient option.

In Denmark, the surveillance system is challenged due to the low prevalence of infected herds. Thus a high temporal *SSe* is needed to detect newly infected herds as soon as possible, so that eventual BVD outbreaks are limited and PIs are removed soon. Therefore, in such a situation a proper early-warning system must be set.

The definition of "early-warning" implies that, time is involved in the evaluation of the system. In previous studies, where scenario trees have been used and surveillance sensitivity has been evaluated (Martin et al., 2007a), it has been suggested that for "slowly" spreading diseases the *SSe* could be evaluated on yearly basis, while for "quickly" spreading diseases, monthly analysis of the *SSe* could be made. Hence, the importance of the High Risk Period (*HRP*) (Horst et al., 1997) or timeliness (Hoinville et al., 2013), was not investigated. We agree on this approach when surveillance is made for highly diffusive diseases, as is the case of Classical Swine Fever in the study by Martin et al. (2007b), because the within herd prevalence at which the system gives a positive result (threshold/design prevalence) should be reached soon after infection. For slowly spreading viruses, as is the case of BVD when introduced to a large herd by

TI animals (manuscript II), the temporal *SSe* has to be estimated. The approach we proposed could be applied for surveillance systems where not all animals are sampled within a herd (see perspectives below) and where the force of infection of the disease is low (low transmission rate and low reproduction ratio).

According to our results, there is no need to replace the blocking ELISA with the SVANOVIR ELISA, if the purpose of the surveillance system is to show on annual basis that the prevalence of herds infected with at least 1 PI calf is <0.2%, since under this scenario the two tests gave similar *SSe* and *PLow* estimates (> 95%).

In contrast, for the other surveillance situations, such as showing complete freedom from PI calves (< 1 herd with 1 PI calf), we found that using the SVANOVIR ELISA on BTM, instead of the blocking ELISA would increase the temporal *SSe* noticeably.

Nonetheless, with any of those tests, the probabilities of detection would be low in the case a TI animal is introduced to the herd. This finding was due to two main reasons: a) usually when a TI animal is introduced, the BVDV spreading dies out (self-clearance), before causing an outbreak (with secondary infected cattle) within the herd, and b) if an outbreak occurs, the time for detection is longer than when a PI animal is introduced into the herd. Hence, overall when a short *HRP* is assumed, detection is more likely to occur in the presence of PIs (as shown in manuscript IV).

This is due to the fact that, PI animals have 16 times higher transmission rate (β within animal groups) than TI animals (Viet et al., 2004; Ezanno et al., 2007; manuscript II). Hence, after introduction of a PI into a naïve herd, the probability that at least one susceptible animal is infected (e.g. in a day) is higher than when a TI is introduced (the within herd BVDV spread is faster in the former case).

Furthermore, if the first introduced PI (or TI) is removed from the herd (e.g. by the farmer), or the first introduced TI seroconverts (end of virus shedding period), the BVDV may remain present in herd in a latent phase (Lindberg and Alenius, 1999), in PI calves carried by Trojan cows. These cows seroconvert and do not shed the virus until calving. After calving (or after

abortion) of a PI calf, Trojan cows could infect herd mates during the first few days (e.g. with BVDV contaminated lochia) (Lindberg et al., 2004). When the PI calves will be born, BVDV spread will start again in the herd mainly by those animals. In manuscript II, this latent phase is shown for the herd we used to validate the model, and is represented by the time period between week 11 and week 29, when new PIs were born in the herd (see figures 2 and 3 in manuscript II).

Further studies could investigate the risk of spreading BVDV to other herds during the *HRP*, or before the BVDV spreading ends within the first infected herd(s) after introduction of a TI animal. Such studies would point out the optimal surveillance frequency that is needed to detect the infected herd before the disease is spread to other herds.

Furthermore, it could be argued that increasing the sampling frequency (assuming shorter *HRP*) would increase the temporal *SSe*. This is the case if the threshold prevalence has already been reached in the milking group. For instance, if the infected herd reported in study II (herd B in study I) had been tested more frequently after week 41, when the threshold prevalence (50%) was reached, then more samples would have been positive (bl% constantly >50%) (see Fig. 3 in manuscript IV, or Fig. 5 above).

In contrast, testing the herd more frequently before the threshold prevalence is reached could be inefficient. This was the case of the last BVDV infected herd detected in Denmark by BTM testing (herd A in manuscript I), where up to 350 cows could be present. In this herd, new animals were bought between 2008 and 2009 from a previously BVD infected herd. We suspect that BVDV was introduced at that time, by cows which aborted PI calves and/or by TI animals. The BTM of the herd showed bl.% around 0 until 5th October 2011. Hence, even if a higher number of BTM samples were tested between 2009 and 2011, the probability that the BTM was classified positive would not have increased (just the costs would have increased due to more testing). After October 2011, when a “signal” was shown in the BTM, both the prevalence of positive milking cows and the bl.% were constantly >50%. Thus, an efficient sampling frequency must be set according to the test used, the herd sizes present in the country, and taking into account for the fact that different patterns of BVDV introduction (PI or TI animals) are possible.

Moreover, the assumed infection status and the design prevalence (P_H *0.2% or 0.02%*) can affect remarkably the temporal *SSe* estimates, the *PLow* and the *PFree*. In our situation, it was rather difficult to know which infection scenario was more likely in Denmark and we decided to evaluate the system under both situations.

Additionally, use of expert opinion was avoided in the scenario trees proposed in study IV (Figures 7, 8, and 9 above) and we based the relative risk estimates (*RR*) on a quantitative risk assessment made for each herd category (with or without import of cattle), by using the model developed in study III. This should have limited the amount of uncertainty due to expert´s personal opinion, which could be sometimes used e.g. to obtain *RR* estimates (Martin et al., 2007a).

CHAPTER 4

Conclusions

The conclusions of this PhD project are:

(1) The SVANOVIR ELISA can detect a lower prevalence of antibody milking cows compared to the Danish blocking ELISA (manuscript I).

(2) The SVANOVIR ELISA appeared to be the significantly "fastest test" between those we considered, in detecting antibodies against BVDV in the BTM (manuscript II).

(3) The herd size affected significantly the lag time between BVDV introduction and detection in BTM using an ELISA (manuscript II).

(4) The main risk of BVD introduction into Danish dairy herds was due to import of live animals from endemic countries. The overall risk can be reduced considerably by testing all imported animals and disinfecting the tools used for hoof trimming (manuscript III).

(5) Using the SVANOVIR ELISA, a higher temporal *SSe* (and related *PFree* and *PLow*) can be achieved. Hence under the current Danish situation it is recommended to use the SVANOVIR ELISA to test the BTM in all Danish dairy herds (manuscript IV).

CHAPTER 5

Challenges and limitations of the studies

Study I

The main challenge in study I was that we found 27 out of 149 cows, which were positive in milk while they were negative in the paired serum. We expected that cows positive in milk were also positive in serum, because antibody titers in serum are usually higher (Caffin and Poutrel, 1988). Mismatch of samples was unlikely. Nevertheless, though rarely, such discrepancies between serum and milk have been reported in the literature. For example, in the study by Niskanen et al. (1989) one out of 55 cows resulted positive in milk but not in serum and 2 out of 84 cows showed higher antibody titres in milk than in serum. This kind of finding is probably due to the different degrees of dilution of the samples (undiluted milk, diluted serum 1:10).

According to Schrijver and Kramps (1998), false positive diagnosis can be made when non-competitive ELISAs (as the SVANOVIR) are used and samples are not diluted before the test is carried out. In that case, positive reactions due to unspecific antibody binding are possible (though a negative control was used in the test, which is meant to correct for this problem). Nevertheless, for our dilution experiments, we did not use samples from the 27 “doubtful cows”, since we considered only animals which were positive in both tests in milk and sera, to be confident that we diluted samples from truly positive cows.

Moreover, we diluted positive samples with negative samples from the same BVD infected herds. Hence some of the negative sample could have arrived from cows, which were in the process of seroconversion, but were still below the cut-off used in milk and serum. If we had used negative milk from BVD negative herds, results could have been different. On the other hand, within infected herds, negative milk will arrive from both kind of animals, those that have not encountered the virus (naïve) and animals that are in the process of seroconversion, but still negative (below the cut-off). Milk from both kind of cows will go to the tank and will dilute antibodies from positive cows. Thus, the way we carried out the experiments is close to reality and to the kind of animals present in infected herds.

Study II

When we developed the stochastic simulation model in R, we assumed that all milking animals produced the same amount of milk and that all seroconverted animals had the same antibody titre in milk. In reality this is not usually the case, and thus, we used a simplification. For instance, the concentration of antibodies in individual milk could be higher at the beginning and at the end of lactation (Niskanen et al., 1989).

Hence, in reality, some infected herds can be detected even if the threshold prevalence is not reached, e.g. when highly positive milking cows are imported to a small herd. For example, Trojan cows could have significantly higher serum antibody titre than other seroconverted animals, which have never carried a PI calf (Brownlie et al., 1998; Lindberg et al., 2001). Therefore, if a Trojan cow is imported to a Danish dairy herd, and such an animal also has high antibody titre in milk, the BTM could give a positive signal before the PI calf is born (e.g. during the latent phase described above). On the other hand, it must be taken into account that, Trojan cows have extraordinary high serum antibody titres in the last 2-3 month of pregnancy (Lindberg et al., 2001) when they are usually dry and do not contribute to the BTM. Additionally, Brownlie et al. (1998) argued that in these cows, serum antibody levels rapidly decrease after calving or abortion of the PI calf.

Thus, our *SSe* estimates could be partially underestimated compared to the special scenarios described above. Further studies should be carried out, in order to estimate the *Se* of the test used on BTM when the threshold prevalence is not reached in the milking paddock, and/or to estimate how the *Se* changes per day, according to the prevalence of Trojan milking cows present in the herd (or according to the daily prevalence of seroconverted milking cows in general).

Another challenge in study II was to validate the model for the dead born calves. Our model predicted that 1 calf (0; 4) was dead born due to BVDV in the herd we used for the validation process. In contrast, the number registered by the farmer appeared to be higher. On the other hand, we did not know if those registered by the farmer were dead born due to BVDV or due to other health problems. We could only see that, in the years when BVDV was present in the herd

(2010-2011), the number of dead born calves registered was higher than the mean of the previous years (see manuscript II).

Study III

In the risk assessment study, we considered the prevalence of virus positive animals within infected herds abroad (WHP in study III). We used this input as a Pert distribution, which was set according to the within herd prevalence estimates we found in literature (Table 1, manuscript III). The highest limit was based on the prevalence reported by Billinis et al. (2005).

We considered the within herd prevalence of Trojan cows, as included in the range of values we used for WHP. In fact, as explained above, Trojan cows have transient viremia within a week from infection, then they become immune lifelong and will not shed virus during pregnancy. Hence, the prevalence of dams carrying PIs should be a value between the extremes we used.

Furthermore, according to Houe and Meyling (1991), the risk of fetal infection during the first 90 days of pregnancy is 3.3%, which is very close to the prevalence of PIs usually found in a herd (Houe, 1999). Moreover, Houe and Meyling (1991) stated that there was no significant difference between the percentage of fetal infections in early pregnancy (3.3%) and the prevalence of live PI animals younger than 1 year (2.9%). The 0.4% difference was suggested to be caused by abortions, or neonatal deaths, or high mortality of PIs. Thus, we consider it reasonable to assume that the prevalence of Trojan cows is similar to the prevalence of live PIs in a herd. Additionally, in our data, the pregnancy status of the imported animals was not available.

Regarding the imported embryos, we could not distinguish between in vivo and in vitro derived embryos. It must be remarked that the risk of contamination with BVDV is lower for in vivo derived embryos than in vitro derived embryos. On the other hand, as stated by Stringfellow and Givens (2000): “sanitary procedures for producing pathogen free, in-vivo-derived embryos are efficacious if the ethical and technical excellence of those performing the procedures can be assured”. The same authors stated that “pathogens found in body fluids or

as contaminants in media might remain in close proximity to the embryo until the time of transfer". Hence, we considered the risk due to in vivo derived embryos as not negligible.

Therefore, when we set the probability that an infected embryo causes viremia in the receiving cow (see PVE in Table 6, manuscript III) we decided to use the same estimates for both kinds of embryos, which could have caused an overestimation of the risk in the case all the imported embryos or part of them, were derived in vivo. Moreover, the probability that an infected embryo caused viremia in the naïve receiving cow was set to 1 according to Gard et al. (2010). So our risk estimates for this mean of BVDV introduction are conservative and represent the worst-risk scenario. Additionally, in (Gard et al. 2010) the amount of BVDV placed with the embryos "was the largest amount of the average range (100 to 450 CCID50/embryo) known to be previously associated (Gard et al., 2009) with individual in vivo-derived and in vitro produced embryos after processing procedures". Thus, our simulations take into account that both kind of embryos can have a high amount of BVDV and can cause viremia. It is important to mention that there is a low chance that this happen, but is not impossible. Additionally, we showed results (as sensitivity analysis) considering a lower probability of viremia based on the study by Waldrop et al. (2004).

Regarding the probability that BVDV survives in contaminated embryos and sera, it must be noted that such a probability can be affected by several unpredictable variables (e.g. time elapsed between embryo collection and transfer, dose of virus present on the embryo during collection, preparation and transfer, technical skills of the personal carrying out the washing procedures etc.). We assumed that under the constant conditions of a cryopreserved embryo, the virus could survive until implantation.

Furthermore, fetal calf serum can be contaminated with BVDV (Bolin et al., 1991), and it can be used as culture media for in vitro produced embryos and for (non-surgical) collection of in vivo produced embryos (Waldrop et al., 2004). In a previous risk assessment, Perry (2007) assumed that the probability that the bovine sera was contaminated was 0%, and that only y-irradiated and heat treated sera was used. We used the same assumption.

As we stated in manuscript III, if guidelines for sterilizing and testing bovine sera were not fully respected, risk due to vaccines and embryos could be higher than we estimated. On the other hand, we found it very difficult to introduce estimates for sera contamination into the embryos´ scenario tree, because: (i) sera batches could be composed of sera collected in different (unknown) areas with different prevalence, (ii) the description of the sterilization procedures used on the calf sera batches was not available in our data and (iii) the production procedures could change with countries.

Regarding imported sheep and goats, we assumed that they did not represent a relevant risk for Danish dairy herds, since the contact between Danish dairy herds and sheep and/or goats is very limited. Moreover, according to data received from the Danish Cattle Federation, only few sheep and goats were imported between 2002 and 2013 (see manuscript III).

For trucks visiting herds abroad for export purposes, data was available with the date of the animal movement and the country of destination. On the other hand, it was unknown which trucks were from Danish exporters and which were from abroad. Neither, we had data on veterinarians and hoof trimmers practicing in Denmark and abroad. For these reasons, efforts were made to obtain information on all the three variables by contacting several experts and institutions. This required a long study period of approximately 6 months. We concluded that more information is needed on the trucks used for animal exports and for animal movements within Denmark. Such lack of data should be limited in the future because during the study period, new legislation has been made and the number plate of the truck should be now registered by Danish dairy farmers.

Regarding the response rates of veterinarians and hoof trimmers to our questionnaire it could be considered as low. To correct for the low response rate and to reduce uncertainty, we suggested to register veterinarians and hoof trimmers practicing abroad.

Study IV

In manuscript IV, the *SSe* estimates were strongly dependent on the probability that the threshold prevalence of antibody positive milking cows was reached on the day of BTM testing (*PTR* parameter). We introduced this parameter in the stochastic scenario trees, because in this way the temporal *SSe* could be estimated. On the other hand, some uncertainty could not be avoided, because the *PTR* value did not match perfectly with the respective herd size and we used a Pert distribution for the *PTR* within each herd category (in the node "Threshold reached ?"). To avoid this problem 1185 scenario trees should have been made, because in 2010, herds had size between 1 and approximately 1185 cows, but this was not feasible.

Furthermore, we assumed that when the threshold prevalence was not reached the herd tested negative, since the probability of detection in our scenario trees (Fig. 7, 8, and 9 above) was based on *PTR*Se* (or *PTR*HSe* when we considered individual serum testing in *ImpoCattle* herds). Unfortunately, as explained above, we did not find any study where daily changes (after BVDV introduction to the herd) in the *Se* of the test used on BTM are reported. Since BVD has been eradicated from Denmark, we could not evaluate the *Se* of the test at the assumed threshold prevalence. For example, we should have compared the BTM test results with the serological testing of the milking animals (by sampling both on the same day: the BTM and the milking cows within several infected herds). This approach was used by Frössling et al. (2006) for *Neospora caninum*.

Therefore, as Graat et al. (2001) did for IBR, we assumed that the test had the default value *Se* reported in literature, if the threshold prevalence was reached.

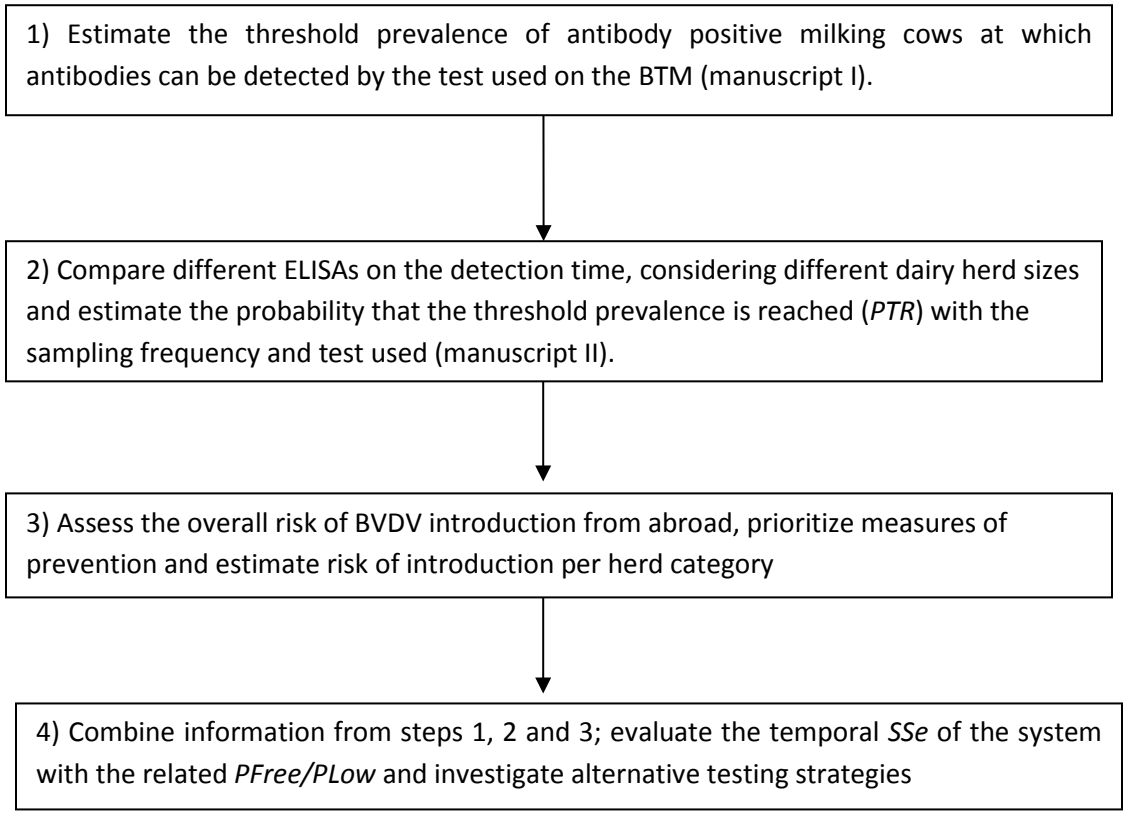
Finally, we also assumed that each herd had a single milk tank. We contacted Arla, which is the largest dairy company in Denmark, who informed us that less than 1% of the dairy herds in Denmark have more than one milk tank. Moreover, when e.g. 2 tanks are present, BVD testing is made using samples from both containers.

CHAPTER 6

Perspectives

6. Perspectives

The approach used in this Ph.D. project can be considered as an example of how the temporal *SSe* of surveillance systems for cattle diseases, which have been eradicated from Danish dairy herds, can be maximized. The final operational diagram is reported here, in four main steps.



For example, if this diagram is applied for infectious bovine rhinotracheitis (IBR), a threshold prevalence of 10-15% (using a blocking ELISA) has been estimated (step 1) (Wellenberg et al., 1998).

In step II, the herd structure proposed in the model developed in study II, can be used to simulate spreading of other viruses within a Danish dairy herd (e.g. such as IBR). The function

simulating introduction of PI animals within a herd must be removed. Then, the parameters reported for BVD in manuscript II, must be found in the literature for IBR and must be fed into the model. Those are: incubation period, viremic period (virus shedding days), days needed to reach seroconversion, and mortality rates in infected animals. Probabilities of abortion according to pregnancy stage and transmission rates within and between groups could be obtained from literature. Additionally, the risk that an animal reactivates virus shedding after becoming immune (latent infections) must be introduced in the model. Such a risk has been estimated by Vonk Noordegraaf et al. (2002).

In step 3, the model developed in study III can be used to carry out the risk assessment for IBR, which can also be transmitted by imports of life cattle, semen, embryos, contaminated trucks and hoof trimmers practicing abroad. In the scenario trees, the quantity of imported goods and their provenience can be maintained from the model of BVD. Then for the branches where probabilities refer specifically to IBR, values can be obtained from the literature or based on expert opinion (e.g. the probability that a truck contaminated with IBR virus causes viremia in a Danish dairy herd).

In step 4, the information obtained in the steps 1, 2 and 3 can be combined in the scenario trees from study IV. The confidence (*PFree*) in complete freedom from IBR (<1 infected herd) or the confidence (*PLow*) in low herd prevalence could then be evaluated with prevalence 0.02% or 0.2% (OIE, 2010 art 11.11.2).

For surveillance of other diseases where individual serum testing is carried out, and not all individuals in a herd are sampled, the same steps can be followed. In this situation, usually the sample size taken within the herd aims to detect at least one infected/immune animal with at least 95% confidence. This means that the study made in manuscript I is not needed, because individual samples are collected and the dilution effect in pooled samples does not need to be studied (as for the BTM in our case). Thereafter, the model developed in manuscript II could be used to investigate the time needed to reach the threshold prevalence within the herd rather than within the milking paddock (as we did for serum testing in *ImpoCattle* herds, in strategies “c” and “d”).

References

Alenius, S., Lindberg, A., Larsson, B., 1997. A national approach to the control of bovine viral diarrhoea virus. In: Proceedings of the 3rd ESVV Symposium on Pestivirus Infections, Lelystad, 19-20 September 1996, The Netherlands, 162-169.

Alvåsen, K., Jansson Mörk, M., Hallén Sandgren, C, Thomsen, P.T., Emanuelson, U., 2012. Herd-level risk factors associated with cow mortality in Swedish dairy herds. J. Dairy. Sci. 95, 4352-4362. http://dx.doi.org/10.3168/jds.2011-5085.

Ames, T.R., 1986. The causative agent of BVD: its epidemiology and pathogenesis. Vet. Med. 81, 848-869.

Antonis, A.F.G., Bouma, A., de Bree, J., de Jong, M.C.M., 2004. Comparison of the sensitivity of in vitro and in vivo tests for detection of the presence of a bovine viral diarrhoea virus type 1 strain. Vet. Microbiol. 102, 131-140.

Baker, J.C., 1990. Clinical aspects of bovine virus diarrhoea virus infection. Rev. Sci. Tech. Off. Int. Epiz. 9, 25-41.

Barkema, H.W., Bartels, C.J., van Wuijckhuise, L., Hesselink, J.W., Holzhauer, M., Weber, M.F., Franken, P., Kock, P.A., Bruschke, C.J., Zimmer, G.M., 2001. Outbreak of bovine virus diarrhea on Dutch dairy farms induced by a bovine herpesvirus 1 marker vaccine. Tijdschr Diergeneeskd 126, 158-165.

Beaudeau, F., Belloc, C., Seegers, H., Assié, S., Pourquier, P., Joly, A., 2001a. Informative value of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of bovine viral diarrhoea virus (BVDV) antibodies in milk. J. Vet. Med. B 48, 705-712.

Beaudeau, F., Belloc, C., Seegers, H., Assié, S., Sellal, E., Joly, A., 2001b. Evaluation of a blocking ELISA for the detection of bovine viral diarrhoea virus (BVDV) antibodies in serum and milk. Vet. Microbiol. 80, 329-337.

Beaudeau F., Assié, S., Seegers, H., Belloc, C., Sellal, E., Joly, A., 2001c. Assessing the within-herd prevalence of cows antibody-positive to bovine viral diarrhoea virus with a blocking ELISA on bulk tank milk. Vet Rec. 149, 236-240.

Becher, P., Orlich, M., Kosmidou, A., König, M., Baroth, M., Thiel, H.-J., 1999. Genetic diversity of Pestiviruses: Identification of novel groups and implications for classification. Virology 262, 64-71.

Barrett, D.J., More, S.J., Graham, D.A., O´Flaherty, J., Doherty, M.L., Gunn, H.M., 2011. Considerations on BVD eradication for the Irish livestock industry. Irish Vet. J. 64, 1-10. http://www.irishvetjournal.org/content/64/1/12

Billinis, C., Leontides, L., Amiridis, G.S., Spyrou, V., Kostoulas, P., Sofia, M., 2005. Prevalence of BVDV infection in Greek dairy herds. Prev. Vet. Med. 72, 75-79.

Bitsch, V., and Rønsholt, L., 1995. Control of bovine viral diarrhea virus infection without vaccines. Vet. Clin. North Am. Food Anim. Pract. 11, 627-640.

Bitsch, V., Houe, H., Nylin, B., Rønsholt, L., 1997. Examination of blood and bulk tank milk samples to monitor the bovine virus diarrhoea infection status of cattle herds. In: Proceedings of the 3rd ESVV Symposium on Pestivirus Infections, Lelystad 19-20 September 1996, The Netherlands, 158-161.

Bitsch, V., Hansen, K.-E.L., and Rønsholt, L., 2000. Experiences from the Danish programme for eradication of bovine virus diarrhoea (BVD) 1994–1998 with special reference to legislation and causes of infection. Vet. Microbiol. 77, 137-143.

Blickenstorfer, S., Schwermer, H., Engels, M., Reist, M., Doherr, M.G., Hadorn D.C., 2011. Using scenario tree modelling for targeted herd sampling to substantiate freedom from disease. BMC Vet. Res., 7-49.

Bolin, S.R., McClurkin, A.W., Coria, M.F., 1985. Effects of bovine viral diarrhea virus on the percentages and absolute numbers of circulating B and T lymphocytes in cattle. Am. J. Vet. Res. 46, 884-886.

Bolin S.R., Matthews P.J., Ridpath, J.F., 1991. Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. J. Vet. Diagn. Invest. 3, 199-203.

Brock, K.V., 2003. The persistence of bovine viral diarrhea virus. Biologicals 31, 133-135.

Brownlie, J., Clarke, M.C., Howard, C.J., Pocock, D.H., 1987. Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann. Rech. Vét. 18, 157-166.

Brownlie, J., Hooper, L.B., Thompson, I., Collins, M.E., 1998. Maternal recognition of foetal infection with bovine virus diarrhoea virus (BVDV)- the bovine pestivirus. Clin. Diagn. Virol. 10, 141-150.

Caffin, J.P., Poutrel, B., 1988: Physiological and pathological factors influencing bovine immunoglobulin G_2 concentration in milk. J. Dairy Sci. 71, 2035-2043.

Cameron, A.R., Baldock, F.C., 1998. A new probability formula for surveys to substantiate freedom from disease. Prev. Vet. Med. 34, 1–17.

Cannon, R.M., 2002. Demonstrating disease freedom-combining confidence levels. Prev. Vet. Med. 52, 227-249.

Cannon, R.M., 2009. Inspecting and monitoring on a restricted budget – where best to look? Prev. Vet. Med. 92, 163-174.

Collett, M.S., Anderson, D.K., Retzel, E., 1988. Comparisons of the pestivirus bovine viral diarrhoea virus with members of the Flaviviridae. J. Gen. Virol. 69, 2637-2643.

Corapi, W.V., Donis, R.O., and Dubovi, E.J., 1988. Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections. J. Virol. 62, 2823-2827.

Coria M.F., McClurkin A.W., 1978. Duration of active and colostrum-derived passive antibodies to bovine viral diarrhea virus in Calves. Can. J. Comp. Med. 42, 239-243.

Decaro, N., Lucente, M.S., Mari, V., Cirone, F., Cordioli, P., Camero, M., Sciarretta, R., Losurdo, M., Lorusso, E., Buonavoglia,C. 2011. Atypical pestivirus and severe respiratory disease in calves, Europe. Emerg. Infect. Dis. 17, 1549-1552.

Depner, K., Bauer, T., Liess, B., 1992. Thermal and pH stability of pestiviruses. Rev. Sci. Tech. Off. Int. Epiz., 11, 885-893.

Duffell, S.J., Sharp, M.W., Winkler, C.E., Terlecki, S., Richardson, C., Done, J.T., Roeder, P.L., Hebert, C.N, 1984. Bovine virus diarrhoea-mucosal disease virus-induced fetopathy in cattle: Efficacy of prophylactic maternal pre-exposure. Vet. Rec. 114, 558-561.

Edwards, S., 1990. The diagnosis of bovine virus diarrhoea-mucosal disease in cattle. Rev. Sci. Tech. Off. Int. Epiz. 9, 115-130.

Ellis, J.A., Martin, K., Norman, G.R., Haines, D.M., 1995. Comparison of detection methods for bovine viral diarrhea virus in bovine abortions and neonatal death. J. Vet. Diagn. Invest. 7, 433-436.

Ezanno, P., Fourichon, C., Viet, A.-F., Seegers, H., 2007. Sensitivity analysis to identify key-parameters in modelling the spread of bovine viral diarrhoea virus in a dairy herd. Prev. Vet. Med. 80, 49-64.

Fray M.D., Paton D.J., Alenius S., 2000. The effects of bovine viral diarrhoea virus on cattle reproduction in relation to disease control. Anim. Reprod. Sci. 60-61, 615-627.

Fredriksen, B., Sandvik, T., Løken, T., Ødegaad, S.A., 1999. Level and duration of serum antibodies in cattle infected experimentally and naturally with bovine virus diarrhoea virus. Vet. Rec. 144, 111-114.

Fritzemeier, J., Haas, L., Liebler, E., Moennig, V., Greiser-Wilke, I., 1997. The development of early vs. late onset mucosal disease is a consequence of two different pathogenic mechanisms. Arch. Virol. 142, 1335-1350.

Frössling, J., Lindberg, A., Björkman, C., 2006. Evaluation of an iscom ELISA used for detection of antibodies to *Neospora caninum* in bulk milk. Prev. Vet. Med. 74, 120-129.

Fulton, R.W., Purdy, C.W., Confer, A.W., Saliki, J.T., Loan, R.W., Briggs, R.E., Burge, L.J., 2000. Bovine viral diarrhea viral infections in feeder calves with respiratory disease: Interactions with *Pasteurella* spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. Can. J. Vet. Res. 64, 151-159.

Gard, J.A., Givens, M.D., Marley, M.S.D., Galik, P.K., Riddell, K.P., Stringfellow, D.A., Zhang, Y., Edmondson, M.A., 2009. Bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced preimplantation bovine embryos following artificial exposure. Theriogenology 71, 1238-1244.

Gard, J.A., Givens, M.D., Marley, M.S.D., Galik, P.K., Riddell, K.P., Edmondson, M.A., Rodning, S.P., 2010. Intrauterine inoculation of seronegative heifers with bovine viral diarrhea virus concurrent with transfer of in vivo-derived bovine embryos. Theriogenology 73, 1009-1017.

Graat, E.A.M., de Jong, M.C.M., Frankena, K., Franken, P., 2001. Modelling the effect of surveillance programmes on spread of bovine herpesvirus 1 between certified cattle herds. Vet. Microbiol. 79, 193-208.

Graham, D.A., McShane, J., Mawhinney, K.A., McLaren, I.E., Adair, B.M., Merza, M., 1998. Evaluation of a single dilution ELISA system for detection of seroconversion to bovine viral diarrhea virus, bovine respiratory syncytial virus, parainfluenza-3 virus, and infectious bovine rhinotracheitis virus: comparison with testing by virus neutralization and hemagglutination inhibition. J. Vet. Diagn. Invest. 10, 43-48.

Hadorn, D.C., Stärk, K.D.C., 2008. Evaluation and optimization of surveillance systems for rare and emerging infectious diseases. Vet. Res. 39-57. DOI: 10.1051/vetres:2008033

Haigh, J.C., Mackintosh, C., Griffin, F., 2002. Viral, parasitic and prion diseases of farmed deer and bison. Rev. Sci. Tech. Off. Int. Epiz. 21, 219-248.

Hoinville, L., 2011. Animal health surveillance terminology final report from the Pre-ICHAS workshop. International Conference on Animal Health Surveillance 1-27.

Hoinville, L.J., Alban, L., Drewe, J.A., Gibbens, J.C., Gustafson, L., Häsler, B., Saegerman, C., Salman, M., Stärk, K.D.C., 2013. Proposed terms and concepts for describing and evaluating animal-health surveillance systems. Prev. Vet. Med. 112, 1-12.

Horst, H.S., Huirne, R.B.M., Dijkhuizen, A.A., 1997. Risks and economic consequences of introducing classical swine fever into The Netherlands by feeding swill to swine. Rev. Sci. Tech. Off. Int. Epiz. 16, 207-214.

Houe, H., 1993. Survivorship of animals persistently infected with bovine virus diarrhoea virus (BVDV). Prev. Vet. Med. 15, 275-283.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet. Microbiol. 64, 89-107.

Houe, H., Meyling, A., 1991. Prevalence of bovine virus diarrhoea (BVD) in 19 Danish dairy herds and estimation of incidence of infection in early pregnancy. Prev. Vet. Med. 11, 9-16.

Houe H., Heron I., 1993. Immune response to other agents of calves persistently infected with bovine virus diarrhoea virus (BVDV). Acta Vet. Scand. 34, 305-310.

Houe, H., Baker, J.C., Maes, R.K., Ruegg, P.L., Lloyd, J.W., 1995. Application of antibody titers against bovine viral diarrhea virus (BVDV) as a measure to detect herds with cattle persistently infected with BVDV. J. Vet. Diagn. Invest. 7, 327-332.

Houe, H., Lindberg, A., Moennig, V., 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. J. Vet. Diagn. Invest. 18, 427-436.

http://www.scotland.gov.uk/Topics/farmingrural/Agriculture/animal-welfare/Diseases/disease/bvd/eradication

http://www.svanova.com/content/dam/internet/ah/svanova/dk_EN/documents/PI%20BVDV%20ind_02.pdf

Hult, L., Lindberg, A., 2005. Experiences from BVDV control in Sweden. Prev. Vet. Med. 72, 143-148.

Joly, A., Fourichon, C., Beaudeau, F., 2005. Description and first results of a BVDV control scheme in Brittany (western France). Prev. Vet Med. 72, 209-213.

Juntti, N., Larsson, B., and Fossum, C., 1987. The use of monoclonal antibodies in enzyme linked immunosorbent assays for detection of antibodies to bovine viral diarrhoea Virus. J. Vet. Med. B 34, 356-363.

Katholm, J., Houe, H., 2006. Possible spread of bovine viral diarrhoea virus by contaminated medicine. Vet. Rec. 158, 798-799.

Kendrick, J.W., Franti, C.E., 1974. Bovine viral diarrhea: decay of colostrum-conferred antibody in the calf. Am. J. Vet. Res. 35, 589–591.

Kramps, J.A., van Maanen, C., van de Wetering, G., Stienstra, G., Quak, S., Brinkhof, J., Rønsholt, L., Nylin, B., 1999. A simple, rapid and reliable enzyme-linked immunosorbent assay for the detection of bovine virus diarrhoea virus (BVDV) specific antibodies in cattle serum, plasma and bulk milk. Vet. Microbiol. 64, 135-144.

Larska, M., Polak, M.P., Riitho, V., Strong, R., Belák, S., Alenius, S., Uttenthal, Å., Liu, L., 2012. Kinetics of single and dual infection of calves with an Asian atypical bovine pestivirus and a highly virulent strain of bovine viral diarrhoea virus 1. Comp. Immunol. Microbiol. Infect. Dis. 35, 381-390.

Larson, R.L., Brodersen, B.W., Grotelueschen, D.M., Husanker, B.D., Burdett, W., Brock, K.V., Fulton, R.W., Goehl, D.R., Sprowls, R.W., Kennedy, J.A., Loneragan, G.H., Dargatz, D.A., 2005. Considerations for bovine viral diarrhea (BVD) testing. Bovine PR 39, 96-100.

Lindberg, A., 2000. Validation of an antibody ELISA used on bulk milk to screen dairy herds for bovine viral diarrhoea virus infection. Proceedings of the 9th International Symposium on Veterinary Epidemiology and Economics, 2000. Available at www.sciquest.og.nz

Lindberg, A.L.E, Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet. Microbiol. 64, 197-222.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Prev. Vet. Med. 72, 55-73.

Lindberg, A., Groenendaal, H., Alenius, S., Emanuelson, U., 2001. Validation of a test for dams carrying foetuses persistently infected with bovine viral-diarrhoea virus based on determination of antibody levels in late pregnancy. Prev. Vet. Med. 51, 199-214.

Lindberg, A., Stokstad, M., Løken, T., Alenius, S., Niskanen, R., 2004. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. Vet. Rec. 154, 463-467.

Lindberg, A., Berriatua, E., Fourichon, C., Mintiens, K., Houe, H., 2006. Epidemiology and risks. In: EU Thematic network on control of bovine viral diarrhoea virus (BVDV). BVDV Control QLRT– 2001-01573., pp. 24–72, http://www.afbini.gov.uk/chs-thematic-network-position-paper-on-bvd-control.pdf

Liu, L., Xia, H., Wahlberg, N., Belák, S., Baule, C., 2009. Phylogeny, classification and evolutionary insights into pestiviruses. Virology, 385, 351-357.

Loehr, B.I., Frey, H.-R., Moennig, V., and Greiser-Wilke, I., 1998. Experimental induction of mucosal disease: consequences of superinfection of persistently infected cattle with different strains of cytopathogenic bovine viral diarrhea virus. Arch. Virol. 143, 667-679.

Mars, M.H., Van Maanen, C., 2005. Diagnostic assays applied in BVDV control in the Netherlands. Prev. Vet. Med. 72, 43-48.

Mars, M.H., Bruschke C.J.M., van Oirschot, J.T., 1999. Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions. Vet. Microbiol. 66, 197-207.

Martin, P.A.J., 2008. Current value of historical and ongoing surveillance for disease freedom: Surveillance for bovine Johne's disease in Western Australia. Prev. Vet. Med. 84, 291–309.

Martin, P.A.J., Cameron, A.R., Greiner, M., 2007a. Demonstrating freedom from disease using multiple complex data sources 1: a new methodology based on scenario trees. Prev. Vet. Med. 79, 71-97.

Martin, P.A.J., Cameron, A.R., Barfod, K., Sergeant, E.S.G., Greiner, M., 2007b. Demonstrating freedom from disease using multiple complex data sources 2: Case study- Classical swine fever in Denmark. Prev. Vet. Med. 79, 98-115.

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Can. J. Comp. Med. 48, 156-161.

Meyling, A., Houe, H., Jensen, A.M., 1990. Epidemiology of bovine virus diarrhoea virus. Rev. Sci. Tech. Off. Int. Epiz. 9, 75-93.

Mintiens, K.M., Zagmutt, F.J., 2006. Risk-Based animal disease surveillance. Vose consulting (www.voseconsulting.com).

Moennig, V., Liess, B., 1995. Pathogenesis of intrauterine infections with bovine viral diarrhea virus. Vet. Clin. North Am. Food Anim. Pract. 11, 477-487.

Moennig, V., Greiser-Wilke, I., 2003. Perspectives on BVD eradication in Germany. Berl Munch Tierarztl Wochenschr (Article in German) 116, 222-226.

Moennig, V., Eicken, K., Flebbe, U., Frey, H.-R., Grummer, B., Haas, L., Greiser-Wilke, I., Liess, B., 2005. Implementation of two-step vaccination in the control of bovine viral diarrhoea (BVD). Prev. Vet. Med. 72, 109-114.

Moerman, A., Straver, P.J., de Jong, M.C.M., Quark, J., Baanvinger, T., van Oirschot, J.T., 1993. A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. Vet. Rec. 132, 622-626.

Niskanen, R., 1993. Relationship between the levels of antibodies to bovine viral diarrhoea virus in bulk tank milk and the prevalence of cows exposed to the virus. Vet. Rec. 133, 341-344.

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. Vet. J. 165, 125-130.

Niskanen, R., Alenius, S., Larsson, B., Juntti, N., 1989. Evaluation of an enzyme-linked immunosorbent assay for detection of antibodies to bovine virus diarrhoea virus in milk. J. Vet. Med. B 36, 113-118.

Niskanen, R., Alenius, S., Larsson, B., Jacobsson, S-O., 1991. Determination of level of antibodies to bovine virus diarrhoea virus (BVDV) in bulk tank milk as a tool in the diagnosis and prophylaxis of BVDV infections in dairy herds. Arch. Virol. (Suppl. 3), 245-251.

Niskanen, R., Lindberg, A., Larsson, B., Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta. Vet. Scand. 41, 93-99.

Niskanen, R., Alenius, S., Belák, K., Baule, C., Belák, S., Voges, H., and Gustafsson, H., 2002. Insemination of susceptible heifers with semen from a non-viremic bull with persistent bovine virus diarrhoea virus infection localized in the testes. Reprod. Dom. Anim. 37, 171-175.

Nuotio, L., Juvonen, M., Neuvonen, E., Sihvonen, L., Husu-Kallio, J., 1999. Prevalence and geographic distribution of bovine viral diarrhoea (BVD) infection in Finland 1993-1997. Vet. Microbiol. 64, 231-235.

OIE, 2004. Bovine viral diarrhoea Chapter, 2.10.6. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. http://web.oie.int/fr/normes/mmanual/A_00132.htm

OIE, 2010. Enzootic bovine leucosis. Chapter 11.9. Article 11.9.2. Terrestrial Animal Health Code.

OIE, 2010. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis. Chapter 11.11. Article 11.11.2. Terrestrial Animal Health Code.

OIE, 2011. Glossary. Terrestrial Animal Health Code.

OIE, 2013. Bovine tuberculosis, Chapter 11.6. Article 11.6.2. Terrestrial animal health code.

Olafson, P., MacCallum, A.D., and Fox, F.H., 1946. An apparently new transmissible disease of cattle. Cornell Vet. 36, 205-213.

Paton, D.J., Christiansen, K.H., Alenius, S., Cranwell, M.P., Pritchard, G.C., Drew, T.W., 1998. Prevalence of antibodies to bovine virus diarrhoea virus and other viruses in bulk tank milk in England and Wales. Vet. Rec. 142, 385-391.

Pellerin, C., Van Den Hurk, J., Lecomte, J., and Tijssen, P., 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260-268.

Perry, G.H., 2007. Risk assessment of transmission of bovine viral diarrhea virus (BVDV) in abattoir-derived in vitro produced embryos. Theriogenology 68, 38-55.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extintion. Vet. Res. 41 (44), 1-14.

Presi, P., Struchen, R., Knight-Jones, T., Scholl, S., Heim, D., 2011. Bovine viral diarrhea (BVD) eradication in Switzerland-Experiences of the first two years. Prev. Vet. Med. 99, 112-121.

Rasmussen, T.B., Uttenthal, Å., Reimann, I., Nielsen, J., Depner, K., Beer, M., 2007. Virulence, immunogenicity and vaccine properties of a novel chimeric pestivirus. J. Gen. Virol. 88, 481-486.

Ridpath, J.F., Bolin, S.R., and Dubovi, E.J., 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205, 66-74.

Rønsholt, L., Nylin, B., Bitsch, V., 1997. BVDV antigen- and antibody blocking ELISA (DVIV) system used in a Danish voluntary eradication program. In: Proceedings of the 3rd ESVV Symposium on Pestivirus Infections, Lelystad 19-20 September 1996, The Netherlands, 150-153.

Rossmanith, W., Deinhofer, M., Janacek, R., Trampler, R., Wilhelm, E., 2010. Voluntary and compulsory eradication of bovine viral diarrhoea virus in Lower Austria. Vet. Microbiol. 142, 143-149.

Schrijver, R.S., Kramps, J.A., 1998. Critical factors affecting the diagnostic reliability of enzyme-linked immunosorbent assay formats. Rev. Sci. Tech. Off. Int. Epiz. 17, 550-561.

Schuppers, M.E., Frey, C.F., Gottstein, B., Stärk, K.D.C., Kihm, U., and Regula, G., 2010. Comparing the demonstration of freedom from Trichinella infection of domestic pigs by traditional and risk-based surveillance. Epidemiol. Infect. 138, 1242–1251.

Sørensen, J.T., Enevoldsen, C., Houe, H., 1995. A stochastic model for simulation of the economic consequences of bovine virus diarrhoea virus infection in a dairy herd. Prev. Vet. Med. 23, 215-227.

Ståhl, K., Alenius, S., 2012. BVDV control and eradication in Europe - an update. Jpn. J. Vet. Res. 60, 31-39.

Ståhl, K., Kampa, J., Alenius, S., Persson Wadman, A., Baule, C., Aiumlamai, S., Belák, S., 2007. Natural infection of cattle with an atypical ´HoBi´-like pestivirus- implications for BVD control and for safety of biological products. Vet. Res. 38, 517-523.

Stärk, K.D.C., 2009. Risk-based surveillance – An approach coming of age? Royal Veterinary College, North Mymms, UK.

Stärk, K.D.C., Regula, G., Hernandez, J., Knopf, L., Fuchs, K., Morris, R.S., Davies, P., 2006. Concepts for risk-based surveillance in the field of veterinary medicine and veterinary public health: Review of current approaches. BMC Health Serv. Res. 1-8.

Stringfellow, D.A., Givens, M.D., 2000. Preventing disease transmission through the transfer of in-vivo-derived bovine embryos. Livest. Prod. Sci. 62, 237-251.

Tegtmeier, C., Stryhn, H., Uttenthal, Å., Kjeldsen, A.M., and Nielsen, T.K., 2000. Seroprevalence of border disease in Danish sheep and goat herds. Acta Vet. Scand. 41, 339-344.

Thurmond, M.C., 2003. Special article: Conceptual foundations for infectious disease surveillance. J. Vet. Diagn. Invest. 15, 501–514.

Uttenthal, Å., Stadejek, T., Nylin, B., 2005. Genetic diversity of bovine viral diarrhoea viruses (BVDV) in Denmark during a 10-year eradication period. APMIS 113, 536-541.

Valle, P.S., Skjerve, E., Martin, S.W., Larssen, R.B., Østerås, O., Nyberg, O., 2005. Ten years of bovine virus diarrhoea virus (BVDV) control in Norway: a cost-benefit analysis. Prev. Vet. Med. 72, 189-207.

Van Oirschot, J.T., Bruschke, C.J.M., van Rijn, P.A., 1999. Vaccination of cattle against bovine viral diarrhoea. Vet. Microbiol. 64, 169-183.

Viet, A.-F., Fourichon, C., Seegers, H., Jacob, C., Guihenneuc-Jouyaux, C., 2004. A model of the spread of the bovine viral-diarrhoea virus within a dairy herd. Prev. Vet. Med. 63, 211-236.

Vilček, Š., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega S., Scicluna, M.T., Palfi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. Virol. 146, 99-115.

Vonk Noordegraaf, A., Nielen, M., Franken, P., Dijkhuizen, A.A., 2002. Simulation modelling of BHV1-control programme at national level, with special attention to sensitivity analysis. Livest. Prod. Sci. 76, 153-170.

Waldrop, J.G., Stringfellow, D.A., Galik, P.K., Riddell, K.P., Riddell, M.G., Givens, M.D., Carson, R.L., 2004. Infectivity of bovine viral diarrhea virus associated with in vivo-derived bovine embryos. Theriogenology 62, 387-397.

Welsh, M.D., Adair, B.M., Foster, J.C., 1995. Effect of BVD virus infection on alveolar macrophage functions. Vet. Immunol. Immunop. 46, 195-210.

Wellenberg G.J., Verstraten, E.R.A.M., Mars, M.H., van Oirschot, J.T., 1998. ELISA detection of antibodies to glycoprotein E of bovine herpesvirus 1 in bulk milk samples. Vet. Rec. 142, 219-220.

Xue, W., Mattick, D., Smith, L., Maxwell, J., 2009. Fetal protection against bovine viral diarrhea virus types 1 and 2 after the use of modified-live virus vaccine. Can. J. Vet. Res. 73, 292-297.

Zimmer, G., Schoustra, W., Graat, E.A.M., 2002. Predictive values of serum and bulk milk sampling for the presence of persistently infected BVDV carriers in dairy herds. Res. Vet. Sci. 72, 75-82.

Zimmer, G.M., Van Maanen, C., De Goey, I., Brinkhof, J., Wetnink, G.H., 2004. The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. Vet. Microbiol. 100, 145-149.

APPENDIX

Manuscript I

Detection of antibodies against Bovine Viral Diarrhoea Virus in bulk milk according to herd size and antibody ELISA used

Alessandro Foddai[a §], Claes Enøe[a], Anders Stockmarr[b], Kaspar Krogh[c], Åse Uttenthal[d]

[a] *Section of Epidemiology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark.*

[b] *Statistics and Data Analysis Section, Department of Applied Mathematics and Computer Science, Technical University of Denmark, Matematiktorvet, DK-2800 Lyngby, Denmark.*

[c] *Knowledge Centre for Agriculture, Cattle, Danish Cattle Federation, Agro Food Park 15, Skejby, 8200 Aarhus N, Denmark.*

[d] *Section for Virology, National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark.*

[§] Corresponding author

E-mail address:

AF: alefo@vet.dtu.dk;

CE: claes_private@hotmail.com

AS: anst@imm.dtu.dk

KK: KSK@vfl.dk

ÅU: aase.uttenthal@gmail.com

Abstract

Background

Bovine Viral Diarrhea (BVD) is considered eradicated from Denmark, and currently, very few (if any) Danish cattle herds could be infected with Bovine Viral Diarrhoea virus (BVDV). The Danish (antibody) blocking ELISA has been successfully used during the Danish BVD eradication program, initiated in 1994. In this study, changes in (i) the Danish dairy herd size and (ii) in the BVD status of the national dairy flock were evaluated, in relation to surveillance of BVD based on antibody detection in bulk milk. We investigated how these changes could affect the performance of the Danish blocking ELISA and of the SVANOVIR®BVDV-Ab indirect ELISA. The latter has been successfully used to eradicate BVD in Sweden.

Methods

Data (2003-2010) on herd size, milk production, bulk milk surveillance and occurrence of viremic animals were analysed. Additionally, the Danish blocking ELISA and the SVANOVIR ELISA were compared using milk and serum samples. The prevalence of antibody positive milking cows that could be detected by each test was estimated, by diluting positive individual milk samples and making artificial milk pools.

Results

During the investigated years, the median herd size increased from 74 (2003) to 127 cows (2010), while the prevalence of BVDV infected herds decreased from 0.51% to 0.02%. Consequently, the daily milk contribution of one seropositive cow to the bulk milk reduced (from 1.61% to 0.95%), and antibody levels in bulk milk decreased at national level. Moreover, we found that testing bulk milk, the SVANOVIR®BVDV-Ab can detect a lower prevalence of

seroconverted milking cows, compared to the Danish blocking ELISA (0.78% vs. 50%). Values in the SVANOVIR®BVDV-Ab better relate to low concentrations of antibody positive milk (R^2 = 94-98%), than values in the blocking ELISA (R^2 = 23-75%). For sera, the two ELISAs performed equally well.

Conclusions

The SVANOVIR ELISA is recommended for analysis of bulk milk samples in the current Danish situation, since infected dairy herds (e.g. due to import of infected cattle) can be detected shortly after BVDV introduction, when only few milking cows have seroconverted. In sera, the two ELISAs can be used interchangeably.

Keywords: Bovine Viral Diarrhoea, bulk milk, antibody ELISA, surveillance

Background

Antibody enzyme-linked immunosorbent assays (ELISA) are commonly used for bulk milk testing of Bovine Viral Diarrhoea (BVD). The level of antibodies against BVD virus (BVDV) in bulk milk relates to the prevalence of BVDV antibody positive cows in the dairy herd [1]. In Denmark, if the bulk milk is classified as positive to antibodies, blood is sampled from 25-30 individual animals to find at least one antibody positive animal (with 95% confidence, assuming a 10% within-herd prevalence) and to confirm the herd infection status. If the herd is confirmed positive, all animals are sampled to find and remove the persistently infected (PI) cattle. Moreover, animal movements are put under restriction until all PI animals have been eliminated from the herd (usually during a one year period from BVDV detection). PI calves are BVDV-infected in the uterus before the 120th day of gestation; they remain chronically infected and may appear healthy but will shed BVDV throughout their lifetime [2; 3]. Acute BVD infections in late pregnancy and after birth cause seroconversion and lifelong immunity [4; 5]

During the study period Danish dairy herds were screened quarterly by bulk milk testing. The Danish blocking ELISA [6; 7] has successfully been used in the national BVD eradication programme [8]. However, due to changes in cattle production since the eradication programme was initiated in 1994 [9; 10] the average herd size has increased, resulting in an increase in the delivery of milk from individual herds. These changes could result in a greater dilution of individual BVDV antibodies in bulk milk.

The prevalence of herds containing viremic animals is expected to be very low in the national dairy herd (≤ 1/4100 herds), and an evaluation of the BVD surveillance system is required to ensure that BVDV positive herds are detected as soon as possible. The test used must detect a

low prevalence of antibody positive cows (e.g. a single animal) and thus low antibody levels, to minimise the rate of false-negative results from the testing of bulk milk. Early detection of newly infected herds (e.g. due to import of infected cattle from abroad), is crucial to control BVD and keep inter-herd spread of BVDV at a very low level.

The aims of this study were: (i) to investigate how changes in the Danish dairy herd size and BVD prevalence from 2003 to 2010 might affect the surveillance based on two antibody ELISAs and (ii) to compare the Danish blocking ELISA [6; 7] and the SVANOVIR®BVDV-Ab ELISA (Svanova Boehringer Ingelheim, Uppsala, Sweden) [1; 11; 12; 13] for detection of BVDV antibodies in milk and sera. Results should lead to recommendations on which ELISA to use, to have an efficient early warning surveillance system for BVD in Danish dairy herds.

Methods

Data

Data collected between 2003 and 2010 were obtained from the Danish Cattle Federation. The dataset contained the central husbandry registration (CHR) ID of the herds, records of milk production (kg/herd/week), the herd size (number of cows/herd/month) and a quantitative account of the antibody level detected by the Danish blocking ELISA (in blocking percentage) in bulk milk samples. The value of Danish blocking ELISA will from here be referred as bl%.

Data on animals positive to BVD virus (e.g. date of birth and date of testing) were also included.

Antibody ELISAs

The Danish blocking ELISA was performed as previously described [6]. For this test, the sensitivity (Se) and specificity (Sp) when applied to individual milk samples have not been estimated. In bulk milk, when the prevalence of infected dairy herds was 26%, estimates of Se and Sp were 100% and 62% respectively, using a cut-off bl% of 50 [14].

Currently, the decision criteria used by the Danish Cattle Federation to consider whether a herd is likely to be positive, based on bulk milk testing, is a rise in the blocking percentage to 50% [7; 9; 14] and/or two consecutive bulk milk samples ≥ 20%. In this study, individual milk, bulk milk samples and milk pools were defined as positive if the bl% was above 0, according to the current antibody levels in the National dairy population (see results). In serum, the Se and Sp are 96.5% and 97.5% respectively, if a cut-off bl% of 50 is used [6].

The ELISA SVANOVIR®BVDV-Ab [1; 11; 12; 13] was performed according to the instructions in the package insert. Responses were calculated as percentage positivity (PP). In individual milk samples, the reported Se and Sp are 95.2% and 100% respectively, using a cut-off PP of 9. In this study, diluted milk samples and artificial pools of milk representing bulk milk were classified as positive if PP was ≥ 2. According to the manufacturer, this value corresponds to a low antibody level in the herd. In serum, the reported Se and Sp are 100% and 98.2% respectively [15], using PP of 15 as indicative of an antibody positive sample.

Milk and serum testing

Individual milk and serum samples were obtained from three Danish dairy herds (A, B and C). Herd A was determined to be a BVDV positive herd (5th October 2011) due to an increase in the bulk milk antibody titre. Herds B and C were bulk milk tested (for five and 30 months, respectively), to assess the antibody levels after removal of PI animals. During the study period (2010-2011), the herd size in herds A, B and C was around 350, 180, and 259 cows, respectively.

To evaluate the impact of fewer but larger herds and of a reduced prevalence of BVDV positive herds on the surveillance system for Danish dairy herds, the Danish blocking ELISA and the SVANOVIR were compared. The minimum prevalence of BVDV antibody positive cows needed (with each test) to detect a positive antibody titre in a bulk milk sample was estimated. Experiments were carried out by a) analysing dilutions of positive individual milk samples and b) analysing artificially made bulk milk samples with a known proportion of positive milk.

Dilution experiments were carried out using individual samples from herd A. In that herd, serum and milk were collected from 303 milking animals. Of these, 149 cows were selected randomly for our study. Thereafter, milk samples from 77 cows that were positive in both ELISAs in milk and serum were divided into three groups: low (L=19 cows), medium (M=38) and highly (H=20) positive, according to the 1st and 3rd quartiles of the bl% in milk (12% and 34%, respectively). This ranking of the samples was based on the blocking ELISA, because, in a previous pilot study we conducted in herd C, we found that milk samples that were positive in the blocking ELISA were also positive in the SVANOVIR, but not necessarily vice versa. Three cows in group L, three cows in group M and four cows in group H were randomly selected between the 77 cows mentioned above. Milk and serum samples from these ten lactating cows were serially two-fold diluted (1/2 up to 1/128) in BVDV antibody negative milk or serum. BVDV negative milk and

serum samples were tested negative in both ELISAs. Likewise, serum samples from the same cows were diluted in negative serum.

Artificial bulk milk samples were made from ten positive and thirty-one negative cows from herds B and C. In this experiment, cows were classified as positive or negative according to bl% in milk. First, a positive milk pool and a negative milk pool were made. Cows contributing to the positive milk pool had milk bl% between 89% and 97%, and serum bl% between 98% and 99%. Secondly, 19 artificial bulk milk samples (5 ml each) were made, using incremental steps of 5 percentage points in the concentration of the positive pool from 10% to 100%. To focus on bulk milk series with low antibody levels, we additionally analysed 12 artificial bulk milk samples with concentrations of positive milk from 2.5% to 30%, with increments of 2.5 percentage points.

Statistical analysis

The freeware R (version 2.13.2, R core development team, 2010) and Excel (Microsoft Office, 2007) were used for data analysis.

To investigate how the herd size and milk production changed during the investigated period, the annual number of milking herds (from January to December), the herd size, the overall national milk production and the daily amount of milk (kg) delivered per herd and per cow, were calculated for all nine years (2003-2010).

The daily milk contribution (in %), of a seroconverted milking cow to the bulk milk, was estimated assuming that (i) all milking cows had similar milk productions and (ii) approximately

17% (minimum 12% and maximum 20%) of the cows present in the herd are dry and do not contribute to the bulk milk. For example, in a herd with 74 cows, we assumed that 62 cows (minimum 59, maximum 65) were milking daily. Hence, the average individual milk contribution to the bulk milk was 1/62= 1.61% (1.54%; 1.70%) (Table 1). The distribution of dry cows used, was based on our knowledge of the Danish dairy industry and fitted the herd structure in herds A, B, and C.

To study changes in the BVD status, the prevalence of herds with viremic animals was estimated by calculating the annual proportion of herds with at least one virus positive animal. Herds which were closed during the year were also considered.

Moreover, the level of immunity against BVDV of the national dairy herd was evaluated using data on antibody detection in bulk milk. Thus, average bulk milk values (in bl%) at national level were investigated for each year.

Finally, for the artificial pools of milk, a simple linear regression model was used to examine the association between the concentration of positive milk in an artificial bulk milk sample (as a dependent variable) and the level of antibodies measured by each ELISA (as an explanatory variable). A log transformation was used for both variables. The coefficient of determination (R^2) was calculated to estimate the variation in the proportion of positive milk in a pooled sample, which can be explained by the values obtained with the ELISA used.

Results

Descriptive statistics on herd size and BVD status in Denmark

Between 2003 and 2010, the number of dairy herds delivering milk during a full year period decreased from 7075 to 4037. The median herd size was 74 cows in 2003 and 127 cows in 2010 (Fig. 1). The two largest herds had 579 and 1185 cows respectively. The overall national milk production remained at the same level, with approximately 4.4 and 4.7 billion kg in the two years respectively (Fig. 1).

The milk yield per cow increased by 3-4 kg during the nine years, but the contribution of a single animal to the daily herd´s production decreased from 1.61% to 0.95% when comparing herds of median size, and from 0.21% to 0.10% when comparing the biggest herds present in 2003 and 2010 (Table 1).

In 2003, the prevalence of herds with at least one BVDV positive animal was 0.51% (39/7731), whereas in 2010 only 0.02% (1/4255) were found to be virus positive. This was due to the import of pregnant cows carrying PI calves (herd B).

In 2003, 31345 bulk milk samples were analysed. Of those, 95% had a bl% below the cut-off 50%. In 2010, 17298 bulk milk samples were tested, 75% of which had a bl% of 0, while the remaining 25% had a median bl% of 5 (3^{rd} quartile = 9%). The maximum value was bl% = 80 in herd B.

Laboratory comparison of BVDV antibody detection in milk and sera by blocking ELISA and SVANOVIR

In herd A, the prevalence of individual antibody positive milk and sera samples detected by the blocking ELISA was 56% and 71%, respectively. Five cows tested positive in milk but not in

serum. The SVANOVIR tested 87% and 69% positive, respectively, since twenty-seven cows tested positive in milk but not in serum. These 27 cows were not considered for the dilution experiments, because for this purpose, only samples from animals positive in both tests, in milk and sera were used (since we wanted to be confident that diluted samples came from truly positive animals).

With bulk milk samples from the field, both ELISAs classified herd B as positive (bl% = 44; PP = 58) 149 days after removal of the last born PI calf. Herd C was classified negative in the blocking ELISA after 503 days (bl% = 0), but still remained positive in the SVANOVIR after 915 days (PP = 13) (Fig. 2.)

In the dilution experiments, two cows from group H were positive in milk in the blocking ELISA at dilution 1/2 (bl% = 4 and 5 respectively), while all ten animals were negative at dilutions ≥ 1/4. The SVANOVIR was positive in all ten milk samples in all dilution steps down to 1/128 (Fig. 3).

In sera, one cow from group M was negative in the blocking ELISA at dilution 1/64 (bl% = 45). The same animal was negative at the same dilution in the SVANOVIR (PP = 14), together with another cow from the same group (PP = 13). The SVANOVIR appeared to be more responsive to the two-fold dilution steps (Fig.4).

Finally, in artificial bulk milk series with 10-100 % BVDV antibody positive milk, the relation between test values and the concentration of positive milk was significant for both tests (P-value < 0.0001). The R^2 was 75% for the blocking ELISA and 98% for the SVANOVIR. In contrast, when analysing pools with 2.5-30% positive milk, there was not a significant relation between

the bl% and the concentration of positive milk (P-value = 0.12). The R^2 was 23% for the blocking ELISA and 94% for the SVANOVIR.

Discussion

The Danish eradication programme was initiated in 1994 when 39% of the herds were expected to contain PI animals and the average herd size was 42 cows [9]. Since then the herd structure has changed and the BVD incidence has decreased. The results from the present study should provide important information on how to update the Danish BVD surveillance system.

As shown in Figure 1, although important changes occurred in the number of milking herds and their size, the overall Danish milk production remained at the same level between 2003 and 2010. There was a slight increase in milk production per cow, though the proportion of the daily contribution of a single animal to the herd´s bulk milk decreased, suggesting that the dilution of individual antibodies was steadily increasing (Table 1). In fact, in Denmark, the increase in the herd size has been quite abrupt (Fig. 1) and if a single antibody positive animal is present in the herd, this is more difficult to be detected by bulk milk testing compared to the past (Table 1).

Furthermore, our epidemiological investigations showed that BVD can be considered an exotic disease in Denmark, because the prevalence of herds with viremic cattle decreased steadily during the investigated years. In the last few years, only sporadic cases have been detected.

The antibody titre in bulk milk decreased at national level and most of the samples did not have a detectable level of antibodies in 2010. For that reason, in our experiments, we used cut-off bl% =

0 to classify a milk sample as positive with the blocking ELISA. Hence, most Danish dairy herds can be considered to be naive to BVDV.

Therefore, to carry out our experiments, we could not use bulk milk samples and individual milk/serum samples from several infected herds. For that reason, we used the dilution experiments and the artificial pools of milk, to investigate the impact of a changed herd structure and antibody dilution level, on the performance of the test used. Moreover, to represent the bulk milk in our experiments, we assumed that all milking animals produced a similar amount of milk and that all seroconverted animals had the same antibody titre. In reality this is not usually the case, and thus, we used a simplification. For instance, the concentration of antibodies in individual milk could be higher at the beginning and at the end of lactation [12] or if a cow carries a PI calf [16]. Nevertheless, we think that our experiments give important information on the comparison of the two tests when used for bulk milk testing. As shown by Graat et al. [17], for infectious bovine rhinotracheitis (IBR), the threshold prevalence of antibody positive milking cows at which the ELISA can classify the bulk milk as positive, is an important parameter to consider, since is affects the detection time and so the performance of the surveillance system as an early warning system.

We found that the SVANOVIR ELISA can classify the bulk milk as positive, with a lower prevalence of seroconverted cows (and thus sooner after BVDV introduction) than the blocking ELISA. Hence, the former could be used to optimize the Danish surveillance system in dairy herds.

In fact, the dilution experiments showed that, in more than half of the herds (with ≤ 128 milking cows), the SVANOVIR could detect one single antibody positive animal, corresponding to an

individual contribution of 0.78% (1/128) to the bulk milk (Fig. 3). On average, one cow can contribute to 0.90-0.99% of the overall herd´s production (Table 1), and therefore most newly infected herds could be detected soon after infection when the number of positive animals is low. In the largest herds, with 129-1043 milking cows and where the herd size is 147 and 1185 cows respectively (considering that at least 12% are dry), two to nine animals should seroconvert to have a positive BVD antibody signal in a bulk milk sample. With the blocking ELISA, at least 50% of the milking cows should be positive (Fig. 3) to find a positive bulk milk sample. This finding was in agreement with the prevalence of positive milking cows in herd A at the time of BVD detection by bulk milk testing.

Furthermore, the linear regression model with the SVANOVIR´s values as x-variable explained 94-98% of the variation in the concentration of positive milk pool used (y-variable), while the model with the blocking ELISA could explain 23-75%. At low concentrations of positive milk (2.5-30%) the explanatory power of both tests was lower and was not significant for the blocking ELISA. Thus, especially when analysing bulk milk from herds with few positive cows, the SVANOVIR relates better to the low prevalence of positive milking cows contributing to the bulk milk.

According to the literature, a low Se in milk is a common problem for blocking ELISAs. For instance, Zimmer et al. [18] found that the Ceditest blocking ELISA tested the bulk milk negative in one out of 25 herds containing PI animals, although such a herd had a high percentage of serum antibody positive cows. In another study [19], the Ceditest ELISA showed a high level of agreement with the Danish blocking ELISA in bulk milk samples. For the blocking ELISA LSI BVD/BD p80, it is known that herds with a bl% $\geq$ 60 four months apart could have a within-herd prevalence of 93% [20]. Therefore, if it is assumed that the Danish ELISA performs

in a similar way as the aforementioned blocking ELISAs, the threshold prevalence (50%) estimated in the dilution experiments to have positive bulk milk appears correct. In this kind of ELISA system, the cause of low Se in milk and high Se in paired serum could be that serum is a well buffered environment, whereas (differently from serum) milk samples can have a low pH due to acidic bacteria that sours the milk. This creates a suboptimal environment for the antigen-antibody binding. Moreover, antibody levels are usually lower in milk than in serum [21].

With the SVANOVIR, in herd A, 27 cows were positive in milk but negative in serum. This was a surprising finding, because the opposite was expected [21]. According to the veterinarians who carried out the sampling, mismatching of milk and serum samples was unlikely, especially since a very high percentage of animals (27/149 = 18%) showed this unexpected result. With the Danish blocking ELISA, this percentage was by far lower (5/149 = 3.4%). Similar results to ours were found in the study by Niskanen et al. [12] in which one cow out of 55 was positive in milk but not in serum in the SVANOVIR, and 2/84 cows showed antibody titres that were higher in diluted milk than in paired diluted serum. Thus, higher positivity in milk compared to sera can sometimes be found, especially when the SVANOVIR is used. With this test, serum is tested after dilution, while milk is tested undiluted. Schrijver and Kramps [22] suggested that when non-competitive ELISAs are used, as is the case with the SVANOVIR, samples should be diluted in a step before analysis to avoid unspecific binding of antibodies. Unspecific binding is a common problem for non-competitive ELISAs, because non-specific antibodies bind to the well and, depending on the washing conditions, they will be detected by the conjugated antibody.

If the SVANOVIR is used as a screening test for bulk milk, a representative testing of Danish herds could be made to assess the proportion of false positive herd reactors, since the

investigation of a herd suspected of having BVDV (based on bulk milk), is conducted by additional testing of individual animals and the cost is about 800-900 euro.

Regarding results from bulk milk samples of herds B and C (Fig. 2), we showed that the SVANOVIR required more time to become negative again after removal of PI animals. At this stage, alternative testing strategies are used in the herd, such testing of serum from young animals (older than 6 months) born after elimination of PIs from the herd [23]. Moreover, while in herd C both tests showed decreasing bulk milk values; in herd B, the SVANOVIR showed an increasing trend after removal of the last born PI, while the Danish blocking ELISA had steadily decreasing values (Fig. 2). These differences between tests could be due to the fact that some more PI was born in the herd and died before being detected by the veterinarians, who carried out the BVDV clearance procedures. The introduction of such a PI could have been signalled in the bulk milk by the SVANOVIR and not by the blocking ELISA.

Finally, while emphasis was placed on the analysis of milk, findings from the analysis of serum were also important. According to our results both tests can be used to analyse serum from new born calves, to test imported cattle (e.g. pregnant cows which could carry PI calves) or to conduct follow-up studies in dairy herds suspected of being infected.

Conclusions

The combination of increased dilution of individual antibodies in bulk milk and decreased BVDV antibody prevalence is a challenge for the surveillance programmes. In countries with large dairy herds and with low BVDV prevalence (e.g. Denmark), the SVANOVIR could be

preferred for an early warning surveillance system based on bulk milk testing, because a lower prevalence of seroconverted milking cows can be detected (compared to the situation where the Danish blocking ELISA is used). Analysis of individual blood could be performed using either of the two ELISAs.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AF carried out some of the ELISAs on milk and serum samples, set down the study design, carried out the statistical analysis and drafted the manuscript. CE participated in the study design, gave advice during the statistical analysis and helped to draft the manuscript. AS gave advice on the sample sizes calculations and for the statistical analysis, and critically revised the manuscript. KK collected the samples from the herds, gave advice about the Danish dairy industry and made a critical revision of the manuscript. ÅU participated in the study design, carried out some of the test in the laboratories, gave advice from the virological/diagnostic point of view and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by the Ministry of Food, Agriculture and Fisheries of Denmark (Grant number 3412-09-02603) and by the Danish Cattle Federation. We are grateful to Grith J. Clausen, Frank Hansen, Erik Rattenborg and Esben Jakobsen for excellent technical assistance and to the farmers for allowing us to sample the herds.

References

1. Niskanen R: **Relationship between the levels of antibodies to bovine viral diarrhoea virus in bulk tank milk and the prevalence of cows exposed to the virus.** *Vet Rec* 1993, **133**:341-344.
2. McClurkin AW, Littledike ET, Cutlip RC, Frank GH, Coria MF, Bolin SR: **Production of cattle immunotolerant to Bovine Viral Diarrhea virus**. *Can J Comp Med* 1984, **48**:156-161.
3. Brownlie J, Clarke MC, Howard CJ, and Pocock DH: **Pathogenesis and epidemiology of Bovine Virus Diarrhoea Virus infection of cattle.** *Ann Rech Vét* 1987, **18**:157-166.
4. Coria MF, and McClurkin AW: **Duration of active and colostrum-derived passive antibodies to Bovine Viral Diarrhea virus in Calves**. *Can J comp Med* 1978, **42**:239-243.
5. Baker JC: **Clinical aspects of bovine virus diarrhoea virus infection.** *Rev sci tech Off int Epiz* 1990, **9**:25-41.
6. Rønsholt L, Nylin B, Bitsch V: **A BVDV antigen- and antibody blocking ELISA (DVIV) system used in a Danish voluntary eradication program.** *In Proceedings of the third ESVV Symposium on Pestivirus Infections: 19-20 September 1996; Lelystad, The Netherlands.* Edited by Edwars S, Paton DJ, Wensvoort G: Central Veterinary Laboratory, Weybridge; 1997:150-153.
7. Bitsch V, Houe H, Nylin B, Rønsholt L: **Examination of blood and bulk tank milk samples to monitor the bovine virus diarrhoea infection status of cattle herds.** *In Proceedings of the third ESVV Symposium on Pestivirus Infections:19-20 September*

1996; Lelystad, The Netherlands. Edited by Edwars S, Paton DJ, Wensvoort G: Central Veterinary Laboratory, Weybridge; 1997:158-161.

8. Uttenthal Å, Stadejek T, and Nylin B: **Genetic diversity of bovine viral diarrhoea viruses (BVDV) in Denmark during a 10-year eradication period.** *APMIS* 2005, **113**:536-541.
9. Bitsch V, and Rønsholt L: **Control of bovine viral diarrhea virus infection without vaccines.** *Vet. Clin. N Am-Food A* 1995, **11**:627-640.
10. Bitsch V, Hansen K-EL, Rønsholt L: **Experiences from the Danish programme for eradication of bovine virus diarrhoea (BVD) 1994-1998 with special reference to legislation and causes of infection**. *Vet Microbiol* 2000, **77**:137-143.
11. Juntti N, Larsson B, and Fossum C: **The use of monoclonal antibodies in enzyme linked immunosorbent assays for detection of antibodies to Bovine Viral Diarrhoea Virus.** *J Vet Med B* 1987, **34**:356-363.
12. Niskanen R, Alenius S, Larsson B, Juntti N: **Evaluation of an enzyme-linked immunosorbent assay for detection of antibodies to Bovine Virus Diarrhoea virus in milk.** *J Vet Med B* 1989, **36**:113-118.
13. Niskanen R, Alenius S, Larsson B, and Jacobsson S-O: **Determination of level of antibodies to bovine virus diarrhoea virus (BVDV) in bulk tank milk as a tool in the diagnosis and prophylaxis of BVDV infections in dairy herds.** *Arch Virol* 1991, (Suppl. 3):245–251
14. Houe H: **Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections.** *Vet Microbiol* 1999, **64**:89-107.
15. **SVANOVIR®BVDV-Ab: Sets the standard for controlling bovine viral diarrhoea virus (BVDV)**

[http://www.svanova.com/content/dam/internet/ah/svanova/dk_EN/documents/PI%20BVDV%20ind_02.pdf]

16. Lindberg A, Groenendaal H, Alenius S, Emanuelson U: **Validation of a test for dams carrying foetuses persistently infected with bovine viral-diarrhoea virus based on determination of antibody levels in late pregnancy**. *Prev Vet Med* 2001, **51**:199-214.
17. Graat EAM, de Jong MCM, Frankena K, Franken P: Modelling the effect of surveillance programmes on spread of bovine herpesvirus 1 between certified cattle herds. *Vet Microbiol* **79**:193-208.
18. Zimmer G, Schoustra W, Graat EAM: **Predictive values of serum and bulk milk sampling for the presence of persistently infected BVDV carriers in dairy herds.** *Res Vet Sci* 2002, **72**:75-82.
19. Kramps JA, van Maanen C, van de Wetering G, Stienstra G, Quak S, Brinkhof J, Rønsholt L, Nylin B: **A simple, rapid and reliable enzyme-linked immunosorbent assay for the detection of bovine virus diarrhoea virus (BVDV) specific antibodies in cattle serum, plasma and bulk milk.** *Vet Microbiol* 1999, **64**:135-144.
20. Beaudeau F, Assié S, Seegers H, Belloc C, Sellal E, Joly A: **Assessing the within-herd prevalence of cows antibody-positive to bovine viral diarrhoea virus with a blocking ELISA on bulk tank milk.** *Vet Rec* 2001, **149**:236-240.
21. Caffin JP, Poutrel B: **Physiological and pathological factors influencing bovine immunoglobulin G_2 concentration in milk.** *J Dairy Sci* 1988, **71**:2035- 2043.
22. Schrijver RS, and Kramps JA: **Critical factors affecting the diagnostic reliability of enzyme-linked immunosorbent assay formats**. *Rev sci tech Off intern Epiz* 1998, **17**:550-561.

23. Houe H, Baker JC, Maes RK, Ruegg PL, Lloyd JW: **Application of antibody titers against bovine viral diarrhea virus (BVDV) as a measure to detect herds with cattle persistently infected with BVDV.** *J Vet Diagn Invest* 1995, **7**:327-332.

Figures:

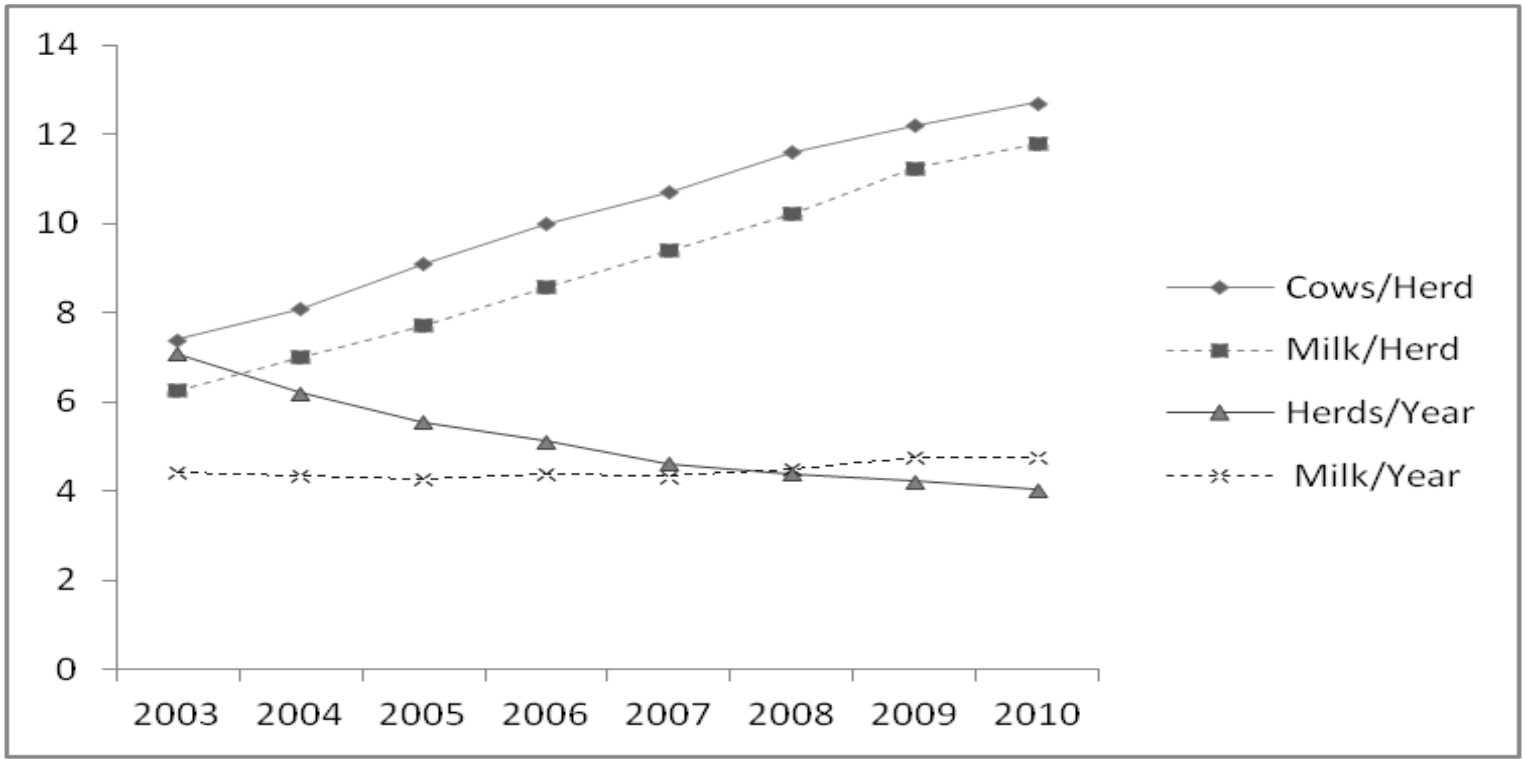


Figure 1 - Changes in herd size and milk production from 2003 to 2010.

Cows/Herd = median herd size (divided by 10); Milk/Herd = milk produced per herd (in 100,000 kg). Herds/Year = number of Danish dairy herds (in 1,000), which delivered milk from January to December; Milk/Year = national milk production (in billion kg of milk).

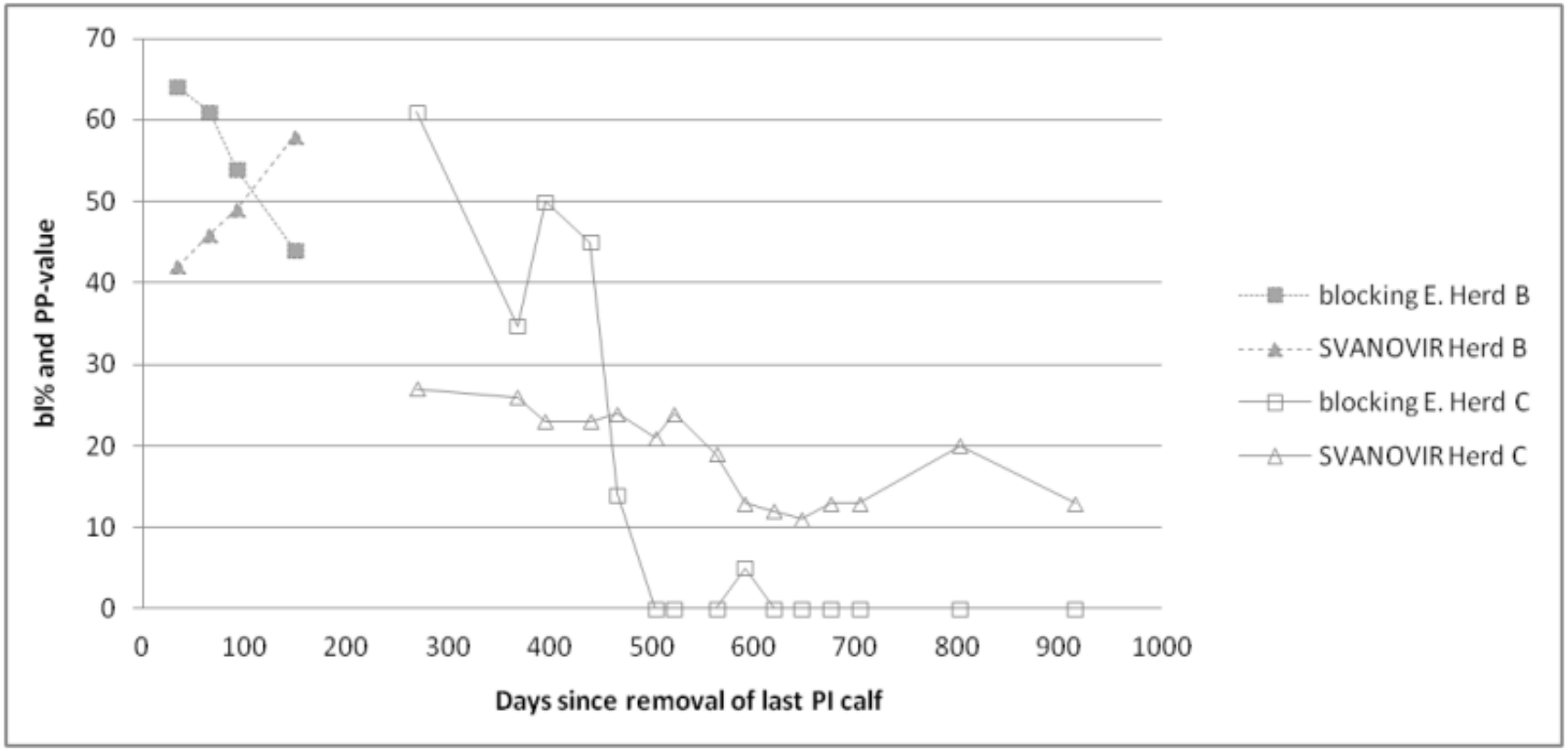


Figure 2 - Change in bulk milk values after removal of last born PI calf (herds B and C).

The y-axis represents the bl% and PP-values according to the blocking ELISA and the SVANOVIR respectively, while the x-axis represents the number of days elapsed between the removal of the last born PI calf and the bulk milk sampling.

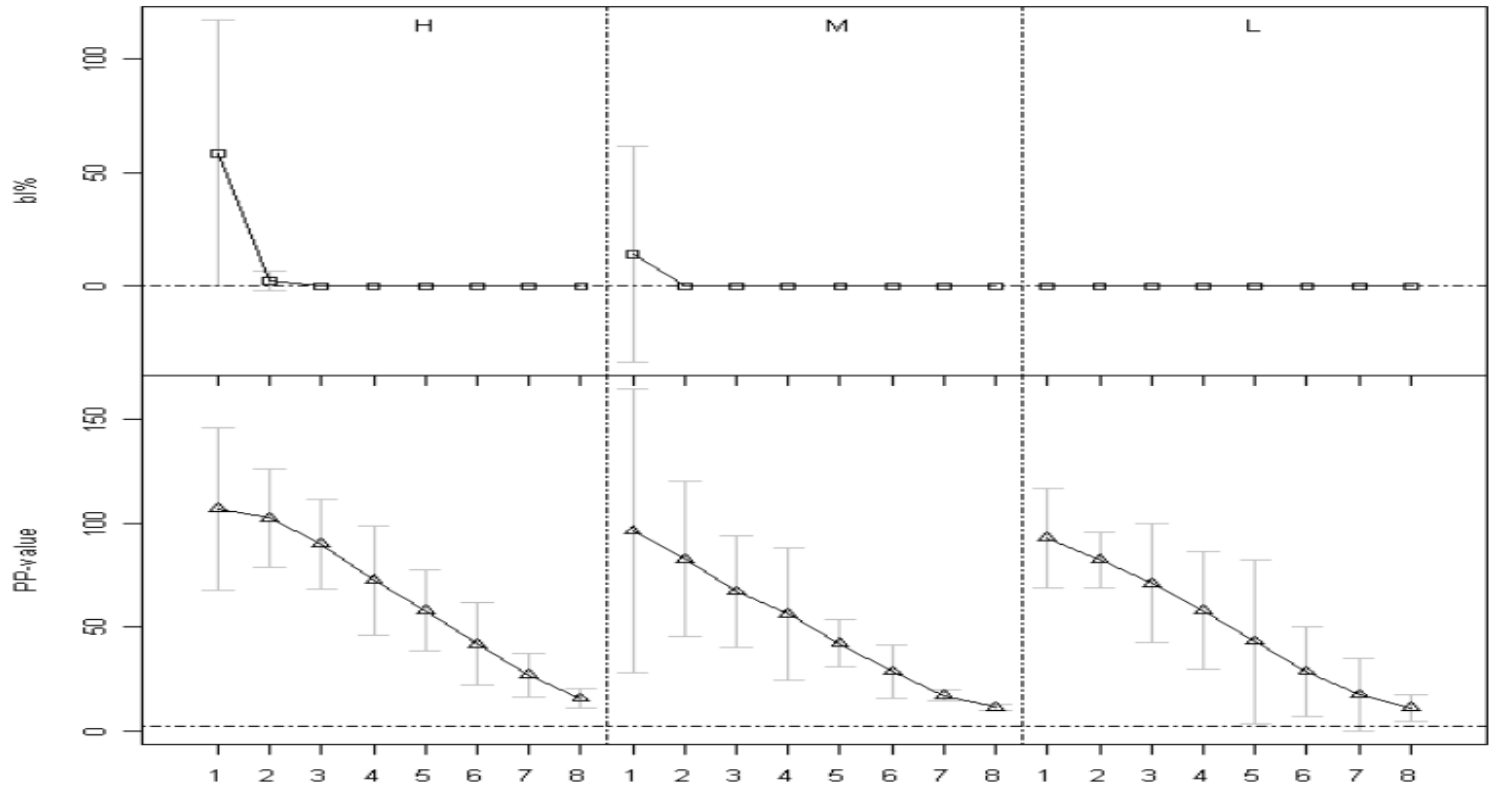


Figure 3 - Results obtained on diluted individual milk samples.

On the x-axis, 1 corresponds to the undiluted sample, while 2-8 represent dilution steps 1/2 up to 1/128. □ = mean bl% with the Danish blocking ELISA; Δ = mean PP-value with the SVANOVIR; grey bars = 95% confidence interval around each mean, H = highly positive group (n=4), M = medium positive group (n=3), and L = low positive group (n=3). Horizontal dashed lines represent the cut-offs (bl% = 0 and PP = 2) at which a milk sample (representing a bulk milk sample in the field) was classified as positive.

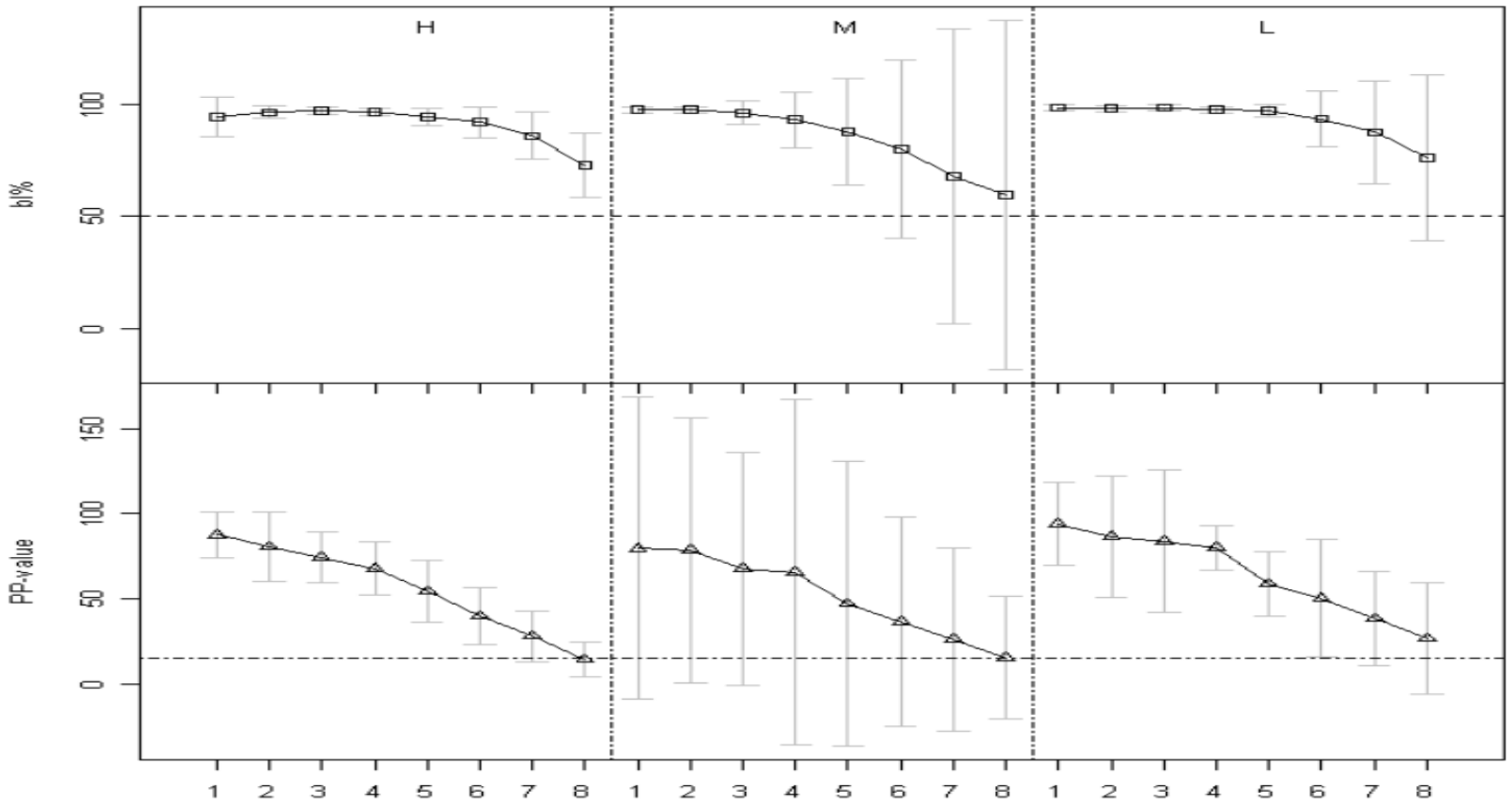


Figure 4 - Results obtained on diluted individual serum samples.

Interpretation as for Figure 3, though in this case, the cut-offs (horizontal dashed lines) for the blocking ELISA and the SVANOVIR are bl% = 50 and PP =15, respectively.

Table 1 - Changes in number of milking cows per herd and their individual contribution to the bulk milk.

a) Number of milking cows/herd/day, b) amount of milk produced (kg) per cow/day and c) daily contribution of a single cow to the bulk milk (in %). Parameters “a”, “b” and “c” were estimated based on our knowledge of the Danish dairy industry and assuming that usually 83% (minimum 80%, maximum 88%) of the cows within the herd are milking.

Parameter	2003			2010		
	80%	83%	88%	80%	83%	88%
a	59 (463)	62 (480)	65 (509)	101 (948)	105 (984)	111 (1043)
b	25 (28)	25 (27)	23 (26)	29 (32)	28 (31)	26 (29)
c	1.70 (0.22)	1.61 (0.21)	1.54 (0.20)	0.99 (0.11)	0.95 (0.10)	0.90 (0.10)

Manuscript II

Preventive Veterinary Medicine xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk

Alessandro Foddai[a,*], Claes Enøe[a], Kaspar Krogh[b], Anders Stockmarr[c], Tariq Halasa[a]

[a] Section of Epidemiology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark
[b] Knowledge Centre for Agriculture, Cattle, Danish Cattle Federation, Agro Food Park 15, Skejby, 8200 Aarhus N, Denmark
[c] Statistics and Data Analysis Section, Department of Applied Mathematics and Computer Science, Technical University of Denmark, Matematiktorvet, DK-2800 Lyngby, Denmark

ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form 7 July 2014
Accepted 7 July 2014

Keywords:
BVD
Simulation model
ELISA
Herd size

ABSTRACT

A stochastic simulation model was developed to estimate the time from introduction of Bovine Viral Diarrhea Virus (BVDV) in a herd to detection of antibodies in bulk tank milk (BTM) samples using three ELISAs. We assumed that antibodies could be detected, after a fixed threshold prevalence of seroconverted milking cows was reached in the herd. Different thresholds were set for each ELISA, according to previous studies. For each test, antibody detection was simulated in small (70 cows), medium (150 cows) and large (320 cows) herds. The assays included were: (1) the Danish blocking ELISA, (2) the SVANOVIR® BVDV-Ab ELISA, and (3) the ELISA BVD/MD p80 Institute Pourquier. The validation of the model was mainly carried out by comparing the predicted incidence of persistently infected (PI) calves and the predicted detection time, with records from a BVD infected herd. Results showed that the SVANOVIR, which was the most efficient ELISA, could detect antibodies in the BTM of a large herd 280 days (95% prediction interval: 218; 568) after a transiently infected (TI) milking cow has been introduced into the herd. The estimated time to detection after introduction of one PI calf was 111 days (44; 605). With SVANOVIR ELISA the incidence of PIs and dead born calves could be limited and the impact of the disease on the animal welfare and income of farmers (before detection) could be minimized. The results from the simulation modeling can be used to improve the current Danish BVD surveillance program in detecting early infected herds.

1. Introduction

Bovine Viral Diarrhea (BVD) is caused by a pestivirus (BVDV) and can result in substantial economic losses in dairy herds (Sørensen et al., 1995; Houe, 1999). The principal sources of infection are the persistently infected animals (PIs) (Houe et al., 1995), which become infected in utero during the first 120 days of pregnancy (Brownlie et al., 1987; Fray et al., 2000). PIs shed BVDV throughout their entire lifetime. Cattle that have been exposed to BVDV subsequently become transiently infected (TI) (Brownlie et al., 1987). After an incubation period of four to seven days, TI cattle become viremic and shed the virus in small amounts, compared to PIs, for approximately two weeks (Baker, 1990; Mars et al., 1999). These animals seroconvert

* Corresponding author. Tel.: +45 27499397; fax: +45 35886001.
E-mail addresses: alefo@vet.dtu.dk, alessandrotula@hotmail.it (A. Foddai).

http://dx.doi.org/10.1016/j.prevetmed.2014.07.007
0167-5877/

two to three weeks after infection and develop a lifelong immunity (Brownlie et al., 1987; Baker, 1990). Some studies indicate that BVDV can circulate within a herd for long periods due to TI animals (Moerman et al., 1993; Moen et al., 2005). However, Niskanen et al. (2000) considered this kind of BVDV spread to be negligible. Moreover, the herd structure can affect the BVDV spread between animal groups within infected herds (Viet et al., 2004; Ezanno et al., 2007; Ezanno et al., 2008).

Surveillance of BVD in dairy herds is usually based on testing for antibodies in BTM samples with follow-up testing of individual blood samples in BTM positive herds. Antibody enzyme linked immunosorbent assays (ELISAs) are preferred because they are considered to be sensitive and cheap (Niskanen, 1993; Kramps et al., 1999; Beaudeau et al., 2001). A general assumption is that the test performance is constant over time and for herds of different sizes, while in reality newly infected herds can be detected only when a certain prevalence of antibody positive milking cows is reached in the herd, as Graat et al. (2001) showed for Infectious Bovine Rhinotracheitis (IBR). The prevalence at which the BTM can be classified as positive represents the threshold parameter (Graat et al., 2001). The time needed to reach the threshold in herds of different size should be estimated.

In Denmark, BVD is considered to have been eradicated since 2005 (Uttenthal et al., 2005). Currently (2014), it is suspected that long time could elapse between new BVDV introduction into a dairy herd and detection of antibodies in bulk milk, because during the past decade the dilution of individual antibodies in the BTM has increased (due to increased herd sizes). For this reason, a higher number of antibody positive milking cows in a herd may be needed in order to be able to detect the disease using an ELISA.

The aims of our study were (i) to determine whether the herd size and ELISA test used for BTM testing would significantly affect the detection time since BVDV introduction (by a PI or a TI animal) into Danish dairy herds, and (ii) to compare the detection times of three antibody ELISAs.

2. Materials and methods

2.1. Simulation model

A stochastic, individual based and dynamic simulation model running with “day” as a discrete time event was developed using the freeware R (R Development Core Team, 2012). The modeling process consisted of: (1) modeling the herd structure, (2) modeling infection spread, and (3) modeling antibody detection using ELISAs on BTM samples. The model was then validated internally using methods from literature (Halasa et al., 2009) and externally using available field data from an infected herd (A). A sensitivity analysis on input parameters was subsequently carried out.

2.1.1. Modeling herd structure

Herd parameters were set based on herd structure data from 2010 obtained from the Danish Cattle Federation. The data, including distributions that were used to represent stochasticity, are synthesized in Table 1. The model was designed to fit the structure of a typical closed Danish dairy herd (where e.g. BVDV introduction could occur due to imported embryos, semen and contaminated trucks/materials). Animal movements and disease transmission patterns in the herd were dependent on the presence of several groups (Ezanno et al., 2008) that were defined by age and lactation length. Three herd sizes were considered in the study: small (70 cows), medium (150 cows) and large (320 cows). These herd sizes were close to the first quartile, the mean and the 95th percentile of Danish herd size.

Table 1
Herd input parameters and distribution used to estimate the detection time, the number of PIs and dead born calves occurring in small, medium and large dairy herds before detection.

Parameter	Value
Herd size (cows, heifers, calves):	
Small	(70, 58, 4)[a]
Medium	(150, 115, 8)[a]
Large	(320, 250, 18)[a]
Culling rate per year for cows	Pert distribution (min = 32%, mode = 38%, max = 43%)[a]
Culling rate per year for heifers	Pert (4, 7, 12%)[a]
Culling rate per year for calves	Pert (5, 12, 17%)[a]
Parity distribution (1st, 2nd, 3rd and 4th)	(31, 27, 22, and 20%)[b]
Percentage of dry cows	Pert (12, 17, 20%)[b]
Age in the heifers group (in days)	(700; 768; 870)[c]
Days of inter-calving per cow between parity 1 and 2	(365, 399, 451)[c]
Lactation length per cow between parity 1 and 2	(305, 339, 391)[c]
Days of inter-calving per cow after parity 2	(370, 391, 456)[c]
Lactation length per cow after parity 2	(310, 331, 396)[c]

[a] Source: Danish data (2010).
[b] As in herd A (which was used to validate the model) and according to our experience.
[c] As the 1st quartile, median, and 3rd quartile of herd A and according to our experience.

The simulated herds included animals in several age groups: calves (aged between 1 and 60 days), heifers (aged between 61 and 900 days) and cows (dry or milking). According to the Council Directive 97/2/EC, no calf should be confined in an individual pen after the age of two months. The average age at first calving was set at 798 days, because usually, Danish Holsteins calve when they are 26.6 months old (Kristensen and Kristensen, 1998, Kaspar Krogh personal communication).

The dry period between two consecutive lactations was set to 60 days. The maximum age that a cow could reach was seven years, according to the age of first calving and lactation length (Table 1). The distributions used for the lactation length, intercalving period, parity and for the percentage of dry cows, are presented in Table 1. Such distributions were found in the infected herd (A) that we used to validate the model (see Section 2.1.4), and according to our knowledge of the Danish cattle industry, they can be generalized to other Danish dairy herds.

It was also assumed that heifers joined the group of dry cows one month before calving. The average culling rates

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

Table 2
Input parameters used in the model for BVDV infected animals.

Information	Parameter	References
Virus shedding (days)	Pert (7,10,14)	Brownlie et al. (1987), Innocent et al. (1997), Viet et al. (2004).
Probability of abortion according to month of pregnancy[a]		
Month 1	13.2%	Hartley and Richards (1988).
Month 2	1.0%	
Month 3	0.2%	
Month 4	0.9%	
Month 5	3.8%	
Month 6	4.0%	
Month 7	4.0%	
Month 8	4.0%	
Month 9	6.0%	
Month 10	6.0%	

[a] Months of 28 days.

per year were 38%, 7% and 12% for cows, heifers and calves, respectively (Table 1).

The sex ratio of new-born calves was assumed to be 50:50. Male calves were assumed to be removed from the herd 14 days after birth.

2.1.2. Modeling the infection processes

The simulated herds were assumed to be fully susceptible to BVDV, because vaccination against the virus is illegal in Denmark and the country is considered either to be free from the infection or has a very low incidence (Uttenthal et al., 2005). Disease transmission between groups within a herd (e.g. from calves to the milking group) was assumed possible through PIs, while disease transmission within groups could take place through both PIs and TI animals (Viet et al., 2004). Disease transmission between groups due to TIs was not modeled, because it was considered negligible as in previous simulation studies (Viet et al., 2004; Ezanno et al., 2008; Courcoul and Ezanno, 2010). BVDV introduction from neighboring herds was excluded.

Since infectious animals could be transiently infected (TI) or chronically infected (PI), a "SIR-like" model was used to simulate virus spread within the herd. Thus, cattle were categorized as susceptible to infection (S), infectious (I_{TI} or I_{PI}), or recovered (R) with a lifelong immunity. PI animals could not recover and remained in status "I_{PI}" all life.

Calves exposed to the virus in utero after 120 days of pregnancy were assumed to seroconvert and have a lifelong immunity. For calves born to immune cows, passive immunity due to colostrum could last between 120 and 240 days (Kendrick and Franti, 1974; Coria and McClurkin, 1978; Houe et al., 1995) and a Pert distribution with mode 180 days (Baker, 1990; Innocent et al., 1997) was used. Susceptible animals exposed to BVDV started shedding the virus after four days from exposure (latency period), while the shedding period was on average 10 days (as in Innocent et al., 1997) (for distribution see Table 2). PIs could shed the virus throughout their lives and could give birth to PI calves (Brownlie et al., 1987).

Disease transmission at a given point in time depended on the number of infectious cattle (PI and TI), their transmission rates (βs), the number of susceptible and the total number of animals.

The daily probability P_j of a new infection on a specific day, for a susceptible animal in group j within the herd was calculated (Eq. (1)). A randomized transmission coefficient for between groups BVDV transmission ($\beta_{PIk,\,j}$) was set to account for daily variation (Eq. (1)). Then, P_j is given by:

$$P_j = 1 - \left[(1 - \beta_{TI} \times I_{TI}/N_j) \times (1 - \beta_{PI} \times I_{PI}/N_j) \times \Pi_{k;k\neq j}(1 - \beta_{PIkj} \times I_{PIk}/N_j)\right] \quad (1)$$

where: β_{TI} = daily contact rate between pairs of animals within group j, times the probability of infection given contact with a TI animal; I_{TI} = number of TI animals within group j on the current day; N_j = total number of animals within group j on the current day; β_{PI} = daily contact rate between pairs of animals within group j, times the probability of infection given contact with a PI animal; I_{PI} = number of PI animals within group j on the current day; $\beta_{PIk,\,j}$ = daily contact rate from individuals in group k to group j, times the probability of infection given transmission from a PI animal located in another group k; I_{PIk} = number of PI animals within group k, which transmits BVDV to group j on the current day.

The model assumes constantly high infection pressure when PIs are present in the herd (Houe and Meyling, 1991), due to both direct contact (within a group) between infectious (PI or TI) and susceptible animals, and indirect contact between one or more PI animals located in group k and susceptible animals located in group j (due to airborne spread and/or any other mean) (Bitsch and Rønsholt, 1995; Mars et al., 1999; Bitsch et al., 2000; Niskanen and Lindberg, 2003). Furthermore, contacts between animals were assumed to occur randomly within a given group j, and randomly with a different transmission rate from any different group k out of n groups, $k = 1, \ldots, n$, $k \neq j$.

The β_{TI} and the β_{PI} were set at 0.03 and 0.50, respectively (Viet et al., 2004; Ezanno et al., 2007). For $\beta_{PIk,j}$, we used a Pert distribution, with minimum value 0.05, mode 0.10 and maximum 0.40. This Pert distribution was used to simulate the daily variability in the amount of virus transmitted between groups.

2.1.3. Mortality and abortions

For the infected animals, mortality probabilities were based on the literature. PI animals had an annual mortality rate of 50% (Houe, 1993; Viet et al., 2004), while TI animals had the same mortality rate as other uninfected cattle, since it is known that most of the times (70–90%) TIs do not show symptoms of BVD (Ames, 1986; Brownlie et al., 1987; Baker, 1990). The latter assumption was also based on the fact that, in the past, only BVDV-1 serotypes have been introduced and detected in Denmark (Uttenthal et al., 2005). BVDV-1 serotypes are less virulent than BVDV-2 serotypes (Pellerin et al., 1994; Ridpath et al., 1994), which have rarely been detected in Europe (Vilček et al., 2001; Uttenthal et al., 2005).

Probabilities of abortion at different stages of pregnancy were considered based on the study by Hartley and Richards (1988) (Table 2). We assumed that cows in milk that had had an abortion before the first half of pregnancy

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

(140 days) were kept in the milking herd for an extra period of time. This period was set equal to the number of days of pregnancy at which abortion occurred, plus days to achieve a new conception (Hartley and Richards, 1988). In the case of pregnant heifers, permanence in the heifer group was also extended, according to time of abortion. Cows and heifers, which aborted in the first half of pregnancy could become pregnant again after 28 (25% probability) or 49 days (50% probability), otherwise they were culled (25% probability). These probabilities were based on our experience and consultations with the Danish Cattle Federation. The likelihood of new conception was lower in the first insemination (at 28 days), due to the time that is needed for the involution of the uterus after an abortion.

If abortion occurred during the second half of pregnancy, we assumed that the cow had a dead-born calf (e.g. due to stillbirth or malformation) (Sørensen et al., 1995). Cows which aborted in the second half of pregnancy had 80% probability to be culled at the end of lactation (Viet et al., 2004).

2.1.4. Information on disease control and history of the herd used for model validation

Danish dairy herds are tested quarterly for antibodies against BVDV in bulk tank milk with the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997). Herds are classified as "suspected of harboring active BVD infection" if a BTM sample shows blocking % (bl%) >50. According to previous studies, the sensitivity and the specificity of the Danish blocking ELISA are 100% and 62% respectively, if a cut-off bl% of 50 is used (Bitsch and Rønsholt, 1995; Houe, 1999; Houe et al., 2006).

When a herd has been classified as BVD infected by BTM testing and at least one animal has been found antibody positive, all animals are tested for antibodies using the Danish blocking ELISA (Rønsholt et al., 1997). All newborn calves must be separated from the rest of the herd as soon as possible, they must be kept in individual boxes (>50 m away) and a blood sample must be taken before receiving colostrum. If antibodies are not detected, serum is tested for BVDV antigens. If antibodies are detected, because the calf received colostrum before sampling, serum is tested for BVDV by PCR (Rasmussen et al., 2007), to avoid of classifying BVDV positive animals as virus negative due to interference of maternal antibodies (which can occur when the antigen ELISA is used). Whenever possible, BVDV positive calves are retested three weeks apart. If seroconversion does not occur and the calf is still viremic (BVDV positive), it is confirmed as PI and removed from the herd. In our study, calves that were registered as BVDV positive, but could be tested only once, were considered PI. On serum, the sensitivity and specificity of the antibody ELISA are 96.5% and 97.5%, respectively, while the antigen ELISA has sensitivity 97.9 and specificity 99.7% (Rønsholt et al., 1997). For the PCR, the sensitivity and specificity are 100% (Internal report, Lindholm laboratories, 2005).

In the herd that was used to validate the model (herd A), BVDV was introduced through the import of live animals (January 2010) from The Netherlands where the disease is known to be endemic. Two PI calves were born from 2 out of 17 imported cows on 2 and 23 February 2010. In this period, 174 cows, 156 heifers and 14 calves were present. The herd was classified as BVD positive in November 2010 (287 days after birth of the first PI calf) due to an increase in the bulk milk antibody titer (bl% = 65). The two PI calves were males and were moved to another farm, at the age of one month. This other farm belonged to the same farmer and was located 200 m away from herd A. In November 2010, both calves tested virus positive twice (three weeks apart) so that the date of BVDV introduction in herd A could be traced back with high confidence.

Between 2003 and 2010, the mean number of dead born calves registered by the farmer in the Danish cattle database was 22 (95% CI, 20; 24), while 23 and 32 were registered in 2010 and 2011, respectively. During the same period, the median number of cows present in the herd was 187 (2.5th percentile = 173; 97.5th percentile = 220).

2.1.5. Model validation

The internal validation of the model was carried out using three methods (Halasa et al., 2009). These were (1) the rationalism method, (2) the tracing method, and (3) the face validity method. When applying the rationalism method, several scenarios were run with different input values, and these were then compared to the outputs in order to check the consistency and credibility of the model. For instance, the model was run without virus introduction and the outputs were checked, to ensure that outbreaks did not occur within herd A and that the size of the small, medium and large herds remained stable. With the tracing method, the individual-animal characteristics (e.g. age, infectious status etc.) were followed over time and the consistency of the outputs was verified. Finally, using the face validity method, a professor in virology (Åse Uttenthal) and two experts from the Danish Cattle Federation (Kaspar Krogh and Erik Rattenborg) were consulted for feedback on the validity of the assumptions, the credibility of the model structure and the model outputs. The three experts participated in the different BVD eradication phases in Denmark and in herd A.

The external validation was performed according to data reported for herd A in Section 2.1.4 and using Eq. (1). At the start of the simulation, the number of animals per group (cows, heifers and calves) was adjusted in the model to represent the simulated herd. The model was iterated 500 times for 730 days. This number of days served to include the birth dates of all PI animals found in the herd. The number of iterations used, was considered sufficient because, when herd A was simulated with 100 or 500 iterations there was not any significant difference (on the number of born PIs, the *P*-value was 0.95 using the Wilcoxon Rank Sum test). The predicted distributions of born PIs and dead born calves were stable, when 100 and 500 iterations were used.

The weekly cumulative number of PI animals born in the herd was compared with the median number of PIs predicted by the model to externally validate the model outcomes. The model was run introducing the first PI on day one and the second on day 21. Both calves were considered to have been removed from the herd after one month of age, because the farmer kept calves, which were not maintained as replacement, in the stable situated far away from the milking paddock, and BVDV is considered able to spread

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

Table 3
Scenarios used on day one of the model for the sensitivity analysis. Days = days the model was run. β_{TI} = within-group transmission rate from transiently infected animals (TI). β_{PI} = within-group transmission rate from persistently infected animals (PIs). $\beta_{PIk,j}$ = between-group transmission rate from PIs. Results are reported as median (2.5th; 97.5th percentiles) "Detection day", number of PIs (PI) and dead born calves (DB). *Z* is the number of iterations (out of 500) in which the threshold prevalence (Threshold) of the Danish blocking ELISA (50% or 30%) was reached.

Scenario	Threshold	Days	β_{TI}	β_{PI}	$\beta_{PIk,j}$	Detection day	*Z*	PI	DB
I[a]	50%	730	0.03	0.50	Pert (0.05, 0.10, 0.40)	301 (226; 564)	491/500	10 (6; 16)	1 (0; 4)
II	50%	730	0.03	1.00	Pert (0.05, 0.10, 0.40)	296 (222; 594)	490/500	10 (6; 15)[c]	1 (0; 5)[c]
III	50%	730	0.03	0.50	Pert (0.025, 0.05, 0.20)	464 (288; 676)[c]	437/500	12 (6; 21)[c]	1 (0; 4)[c,d]
IV	50%	730	0.03	0.50	Pert (0.10, 0.20, 0.50)	252 (87; 333)[c]	500/500	10 (2; 14)[c]	1 (0; 4)
V	50%	730	0.002[b]	0.50	Pert (0.05, 0.10, 0.40)	354 (246; 635)[c]	472/500	10 (6; 16)	1 (0; 4)
VI	50%	730	0.45	0.50	Pert (0.05, 0.10, 0.40)	39 (34; 47)[c]	500/500	2 (2; 2)[c,e]	1 (0; 5)[c]
VII	50%	365	0.03	0.50	Pert (0.05, 0.10, 0.40)	279 (222; 357)[c]	363/500	10 (6; 14)	1 (0; 4)
VIII	50%	1095	0.03	0.50	Pert (0.05, 0.10, 0.40)	301 (226; 587)	497/500	10 (6; 16)	1 (0; 4)
IX	30%	730	0.03	0.50	Pert (0.05, 0.10, 0.40)	148 (53; 455)[c]	495/500	2 (2; 10)[c]	0 (0; 3)[c]

[a] Baseline scenario (herd A).
[b] Within group transmission rate for TI animals by Cherry et al. (1998).
[c] Significant difference from the baseline scenario (*P*-value < 0.05).
[d] N.B. estimates were rounded to the closest integer and the Wilcoxon Rank Sum test showed if there was a significant difference in the distributions of the two scenarios.
[e] Detection occurred before birth of new PIs (the 2 PIs in this case are those that we introduced on day 1 and 21).

by air up to 40 m (Mars et al., 1999; Bitsch et al., 2000). The model simulated this process mechanistically, in which these two PI calves were moved out of the herd (at day 30 and 51), and thus did not affect the probability of infection anymore. Isolation of other calves at birth started on day 288 to represent the control measures applied by the Danish Cattle Federation after antibody detection by BTM testing (see above).

The prevalence of seroconverted milking cows predicted by the model (within herd A) was compared with the BTM values recorded by the Danish Cattle Federation.

For the antibody detection time, the external validation was performed comparing the predicted days to the time observed in the field using the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997). For that purpose, the model was run without the control measures. The simulation stopped when the threshold prevalence of antibody positive cows, which could be detected by the Danish ELISA (50%, see below), was reached within the milking herd.

Moreover, we compared the predicted number of dead born calves and cows present in the herd during the simulated period with the data registered by the farmer in the national database (during 2010 and 2011).

2.1.6. Detection time

Three serological assays used in Europe to determine BVD herd status by BTM testing were compared regarding the antibody detection time, defined as the time elapsed between day of BVDV introduction into a herd and day on which the threshold prevalence of seroconverted cows was reached within the milking group. For estimating the detection time of each ELISA, the model was run with 500 iterations, for three years per iteration and for each herd size (Table 1). We considered a maximum running time of three years acceptable, because if the infection is not detected, then the virus could spread to other herds (e.g. by animal movements) and BVD could become endemic in the country.

The tests considered were the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997), the SVANOVIR®BVDV-Ab ELISA (Svanova Boehringer Ingelheim, Uppsala, Sweden) (Juntti et al., 1987; Niskanen, 1993) and the indirect ELISA Pourquier (BVD/MD p80 milk ELISA test, Institut Pourquier) (Beaudeau et al., 2001). The first two ELISAs were chosen because they were used in the eradication of BVD in Denmark, Sweden, Norway and Finland (Bitsch and Rønsholt, 1995) while the latter was included because it is used in countries in which BVD is known to be endemic (e.g. France) (Beaudeau et al., 2001).

For the Danish blocking ELISA, the threshold prevalence was set at 50%, based on a previous pilot study that we made in the Lindholm laboratories (Denmark), and where individual positive milk samples were serially diluted in negative milk samples. A threshold prevalence of 30% was also used, because in another study, the Danish blocking ELISA showed high agreement with the Ceditest blocking ELISA, which is used in The Netherlands (Kramps et al., 1999). Zimmer et al. (2002) found that with the Ceditest ELISA, 1 out of 25 herds harboring PIs showed a false negative BTM value despite 26% of cows in parity one and 36% of cows in other parities were antibody positive. Hence, we considered it reasonable to estimate the detection time also with threshold prevalence 30%.

For the SVANOVIR ELISA and for the indirect ELISA Pourquier the threshold prevalence was set at 6% (Niskanen, 1993) and 9% (Beaudeau et al., 2001), respectively.

The median antibody detection time (in days with 2.5th and 97.5th percentiles), as well as the total number of PIs and dead born calves occurring in the herd (before detection), was compared between tests (considering two ELISAs at a time). The model outputs were compared using the Wilcoxon Rank Sum test in the statistical software R (R Development Core Team, 2012).

2.2. Sensitivity analysis

A sensitivity analysis was performed with input parameters shown in Table 3. In this section, we investigated which scenario gave the output that better fitted the data from herd A.

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

The baseline scenario (Table 3, I) was validated using the structure of herd A (Section 2.1.5). Runs were made for 730 days and iterated 500 times. In the baseline scenario, the threshold prevalence was 50% (Danish blocking ELISA) and the same transmission rates reported in Section 2.1.2 were used.

In the other scenarios, one transmission rate (scenarios II–VI), or the running days (scenarios VII and VIII) or the threshold prevalence of the Danish blocking ELISA (scenario IX) were changed. The Wilcoxon Rank Sum test was used to test whether there was a significant difference (in the predicted detection time, number of born PIs and dead born calves) between the baseline simulation scenario and each alternative scenario. Moreover, the probability of antibody detection was estimated according to the inputs used (Table 3, Z).

In scenario II, the β_{PI} was doubled, to investigate whether a higher within group transmission rate for PI animals could have any significant effect on the output.

In scenario III and IV different triplets of values were used for $\beta_{PIk,j}$. In the former scenario, all values were halved, while in scenario IV the minimum and the mode were doubled and the maximum was set at 0.50 (equal to the β_{PI}). In this way we could investigate the impact of the most uncertain parameter, namely $\beta_{PIk,j}$.

In scenarios V and VI, the transmission rate of TI animals was lowered according to (Cherry et al., 1998) and increased of 15 times, respectively.

In scenario VII, the model was run for 365 days, while in scenario VIII, 1095 days were used to investigate if the surveillance period could affect significantly the detection time.

Moreover, the detection time was estimated using threshold prevalence 30% for the Danish blocking ELISA (scenario IX), to evaluate if our assumption (50% of the milking cows must have seroconverted to detect antibodies in the BTM) was correct.

3. Results

3.1. Validation

According to the rationalism method, we verified that the different herd sizes remained stable when BVDV was not introduced. During a period of three years, the median herd sizes were 72 (2.5th percentile = 60; 97.5th percentile = 88), 148 (130; 174) and 318 (283; 373) cows, in the small, medium and large herds, respectively. In herd A, where BVDV was introduced, the predicted median size was 186 (171; 212) cows during the two simulated years. Those results were considered realistic by the experts we consulted and fitted the data from 2010 to 2011.

To perform the external validation, the cumulative number of born PI animals predicted by the model, was compared to the cumulative number of born PIs found in herd A (29). The overall predicted number was 27 (7; 54). As shown in Fig. 1, the model predicted an increasing trend (black line = median), which was similar to that observed in herd A (red line). On the other hand, before week 41 (when the BTM was found positive), the model predicted a higher number of PIs compared to the data (Fig. 1).

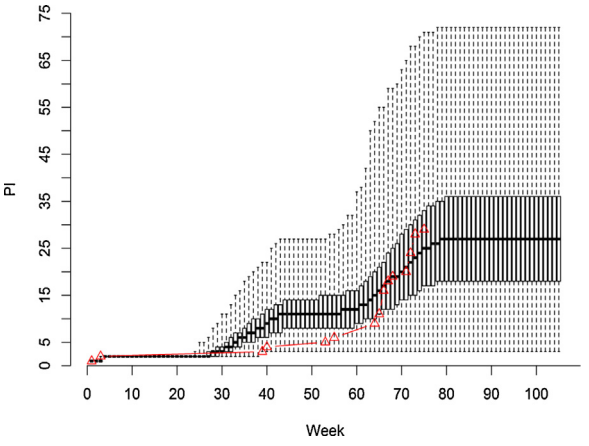


Fig. 1. The predicted cumulative number of born PIs (per week) in herd A, shown in a box-plot (black line = median; bars = 1st and 3rd quartiles, dashed lines = minimum and maximum). The red line represents observed data. The red triangles represent the weekly cumulative number of born PIs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Detection occurred in 491/500 iterations and the predicted detection time was 301 days (226; 564), which was close to that observed in herd A (287 days).

The predicted prevalence of TI milking cows is shown in Fig. 2. The model predicted a peak between weeks 1 and 11. Subsequently, the median prevalence became zero between weeks 11 and 29, while a second peak occurred between weeks 29 and 44 (Fig. 2, black line). Indeed, in Fig. 2, it appears that the virus shedding within the milking group follows an epidemic pattern, which is mainly driven by the incidence of PIs in the herd (Fig. 1). In fact, two new PIs were born at around week 40 (Fig. 1, red line), when a second peak of viremic milking animals was also predicted by the model (Fig. 2). After week 41, virus spread within the milking group ceased, due to the control measures applied by the Danish Cattle Federation after detection (Section 2.1.4 and Section 2.1.5).

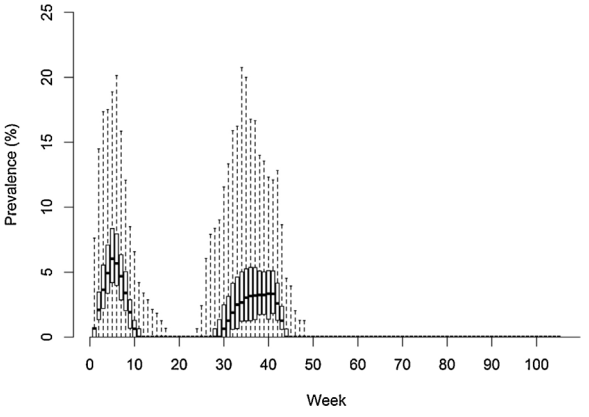


Fig. 2. Box-plots representing the predicted weekly prevalence of viremic (TI) milking cows in herd A. The first peak between weeks 1 and 11 is caused by the two PIs we introduced mechanistically at days 1 and 21. The second peak (weeks 29–44) is caused by the new born PIs.

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

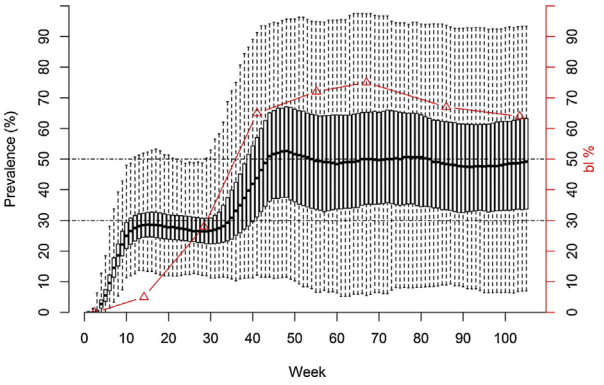


Fig. 3. Box-plots representing the predicted weekly prevalence of antibody positive milking cows in herd A (black line) and the BTM values (red triangles) registered in the database of the Danish Cattle Federation (values in blocking % according to the Danish blocking ELISA, see right axes). The horizontal dashed lines represent the threshold prevalence (30 and 50%), at which the BTM was expected to be classified as positive (bl% > 50). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A comparison between the predicted prevalence of antibody positive milking cows (black line) and BTM antibody values (red line) recorded by the Danish Cattle Federation is shown in Fig. 3. The median predicted prevalence increased between weeks 1 and 11, and remained at a plateau level between weeks 11 and 31. Thereafter, the prevalence started to increase again from week 31 onward. After week 41 the simulated prevalence and the BTM values remained more or less stable. At the end of the two simulated years, the prevalence of seroconverted milking cows was 49% (2.5th percentile = 13; 97.5th percentile = 86%).

The predicted number of dead born calves was 1 (0; 4), which was lower than the number reported by the farmer.

3.2. Detection time for each herd size and assay

The simulated detection time, for each of the three ELISAs and herd sizes are shown in Table 4, while the predicted number of PIs and dead born calves (occurred before detection) is presented in Table 5. The results are shown when one PI calf or one TI milking cow is introduced into a naïve herd.

Using the same test and considering the same BVDV introduction route (PI calf or TI cow) the smaller the herd size is the significantly faster antibodies against BVDV can be detected in BTM samples (Table 4). For instance, the median detection time was approximately three times longer in a large herd than in a small herd, when a PI calf was introduced and the SVANOVIR ELISA was used (Table 4).

On the other hand, if we compare results obtained with the same test, but in different BVDV introduction pathways (PI calf vs. TI cow), detection can occur significantly earlier in a larger herd when a PI calf is introduced (Table 4). For example, introducing a PI calf in a large herd and using the SVANOVIR ELISA, the median detection time was almost twice shorter than in the case when one TI milking cow was introduced to a small herd (Table 4).

The detection time for the SVANOVIR ELISA was generally shorter than for the other ELISAs. The difference between tests was statistically significant in almost all scenarios (Table 4) and the Danish blocking ELISA had the longest detection time in both cases, when threshold prevalence 50% or 30% was used (Table 4). In the latter case, the detection time could be shorter of few months, compared to when threshold 50% was assumed (Table 4).

Table 4
Detection time (according to iterations where the threshold was reached) for: Danish blocking ELISA with threshold prevalence 50% (Blocking_50) or with threshold prevalence 30% (Blocking_30), indirect BVD/BD p80 ELISA (Pourquier), and SVANOVIR ELISA (SVANOVIR) in three herd sizes (small, medium and large). Results are reported as median while between brackets are the 2.5th and 97.5th percentiles.

ELISA	Small = 70	Medium = 150	Large = 320
PI introduced[a]			
Blocking_50	229 (93; 651)	316 (174; 735)	407 (257; 823)
Blocking_30	127 (54; 571)	248 (96; 640)	321 (168; 740)
Pourquier	45 (25; 859)	83 (36; 837)	163 (60; 557)
SVANOVIR*	37 (21; 824)	59 (30; 868)	111 (44; 605)
TI introduced[b]			
Blocking_50	519 (325; 931)	525 (343; 900)	566 (390; 883)
Blocking_30	491 (252; 919)	434 (279; 882)	488 (300; 801)
Pourquier	263 (41; 559)	315 (201; 879)	321 (228; 690)
SVANOVIR	219 (25; 530)	279 (198; 747)	280 (218; 568)

[a] All comparisons between pairs of tests on antibody detection time were statistically significant (P-value <0.05).

[b] All comparisons between pairs of tests on antibody detection time were statistically significant (P-value < 0.05), except for SVANOVIR vs. Pourquier ELISA in large herds (P-value= 0.22) and for Blocking_50 vs. Blocking_30 in small and large herds (P-value = 0.14 and 0.06, respectively).

Using the blocking ELISA the median number of born PIs ranged between 1 (small herds) and 14 (large herds) when one PI calf was introduced (Table 5). Using the same test, the median number of dead born calves ranged between 0 (e.g. small herd) and 2 (large herds) (Table 5).

Using the SVANOVIR and the Pourquier ELISA the median number of PIs ranged between 1 and 2 (Table 5), while the median number of dead born calves was 0.

3.3. Sensitivity analysis

Table 3 shows the results of the sensitivity analysis as predicted detection time, number of born PIs, dead born calves and probability of antibody detection. When the within-group transmission rate of PI animals was doubled (Table 3, scenario II) the number of born PIs and dead born calves were significantly different from the baseline scenario (Table 3, I).

When the between-group transmission rate of PI animals was halved (Table 3, scenario III) the detection time, the number of born PIs and the number of dead born calves were significantly different from the baseline scenario (Table 3, I). By increasing the same transmission rate (Table 3, scenario IV), the number of dead born calves was not significantly different.

When the within group transmission rate of TI animals was lowered (Table 3, scenario V) only the detection time differed significantly from the baseline scenario, while when the same transmission rate was increased (Table 3, scenario VI), all three outputs differed significantly from scenario I.

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

Table 5
Total number of born PIs (PI) and dead born calves (DB) occurring in the herd after introduction of one PI calf or one TI milking cow. Blocking_50 = Danish blocking ELISA with threshold prevalence 50%, Blocking_30 = Danish blocking ELISA with threshold prevalence 30%, Pourquier = indirect BVD/BD p80 ELISA and SVANOVIR = SVANOVIR ELISA. Medians (2.5th and 97.5th percentiles) are reported for three herd sizes (small, medium and large).

	Small		Medium		Large	
ELISA	PI	DB	PI	DB	PI	DB
PI introduced[a]						
Blocking_50	2 (1; 7)	0 (0; 2)	5 (1; 13)	1 (0; 3)	14 (6; 22)	2 (0; 6)
Blocking_30	1 (1; 5)	0 (0; 1)	2 (1; 7)	0 (0; 2)	6 (1; 14)	1 (0; 4)
Pourquier	1 (1; 2)[b]	0 (0; 1)[c]	1 (1; 4)	0 (0; 1)	1 (1; 5)[c]	0 (0; 2)
SVANOVIR*	1 (1; 2)[b]	0 (0; 1)[c]	1 (1; 3)	0 (0; 1)	1 (1; 4)[c]	0 (0; 2)
TI introduced						
Blocking_50	2 (1; 9)[e]	0 (0; 1)[e]	5 (1; 11)	1 (0; 2)	12 (4; 24)	2 (1; 5)
Blocking_30	2 (1; 5)[e]	0 (0; 1)[d,e]	2 (1; 5)[d]	0 (0; 1)	4 (1; 15)	2 (0; 4)
Pourquier	1 (0; 2)	0 (0; 1)[c,d]	2 (1; 4)[c,d]	0 (0; 1)[c]	2 (1; 4)[c]	0 (0; 2)[c]
SVANOVIR	1 (0; 2)	0 (0; 1)[c]	1 (1; 3)[c]	0 (0; 1)[c]	1 (1; 4)[c]	0 (0; 1)[c]

[a] All comparisons between pairs of tests were statistically significant (P-value < 0.05).
[b] N.B. estimates were rounded to the closest integer and the Wilcoxon Rank Sum test showed if there was a significant difference in the distributions of the two scenarios.
[c] No significant difference between SVANOVIR and Pourquier (P-value > 0.05).
[d] No significant difference between Blocking_30 and Pourqueir.
[e] No significant difference between Blocking_50 and Blocking_30.

By running the model for less than two years (scenario VII), the detection time was significantly different from the baseline scenario (Table 3). Instead, running the model for three years (scenario VIII) no significant difference was found with the baseline scenario (Table 3).

With threshold prevalence 30% (Table 3, IX), outputs were significantly different from those of the baseline scenario (where 50% was used).

The probability of reaching the threshold prevalence within the simulated period (Table 3, Z), was similar between scenario I and II (Table 3). When the $\beta_{PIk,j}$ or β_{TI} were increased (Table 3, scenario IV and VI) the probability of detection was higher than in the baseline scenario, while when any of those transmission rates was reduced, detection occurred in a lower proportion of iterations than in the baseline scenario (Table 3, scenarios III and V). If the running time was reduced from 730 to 365 days, detection was by far less likely to occur than in the baseline scenario (Table 3 scenario I vs. scenario VII). Instead using 1095 days or threshold prevalence 30% (scenario VIII and IX) the probability of detection was slightly higher compared to the baseline scenario (Table 3, I).

4. Discussion

4.1. Model validation

According to the rationalism method, the herd size was replicated correctly, when BVDV was introduced (in herd A) and without introducing the virus (for the small, medium and large herds). We considered a variation of ±2 animals in the median number of cows during a running period of 2–3 years to be an acceptable level of variation. Moreover, the fluctuations of the number of cows were considered realistic by the experts we consulted. Regarding the external validation, only few published BVDV transmission models have been validated using field data (Sørensen et al., 1995; Viet et al., 2004; Viet et al., 2007).

The two curves representing the predicted number of born PIs and the actual cumulative number of PIs were rather similar (Fig. 1). Lindberg and Alenius (1999) suggested that PIs occur in a herd in different cycles. In each cycle, an increasing number of PIs can be observed, making the infection display a "two- or three-stage rocket"-type of pattern (Lindberg and Alenius, 1999). The prediction of the model (Fig. 1) was in agreement with the waves of PIs observed in herd A in weeks 40–54 and between weeks 65 and 75, which is consistent with Lindberg and Alenius (1999). Additionally, the final predicted number of PIs was close to the number observed in the field. The higher number of PIs predicted by the model before week 41 (before the BTM was found positive) (Fig. 1), could be caused by the fact that some of the PIs could have been born before detection and thus died or were removed by the farmer. According to Houe (1993), the annual risk rate of a PI dying or being removed from a dairy herd due to unthriftiness is 50%.

The epidemic pattern of TI milking cows (Fig. 2), related to the incidence of born PIs in the herd (Fig. 1), is in agreement with literature, where it is suggested that the main sources of infection within the herd are the PI animals (Lindberg and Alenius, 1999; Niskanen et al., 2000). Thus, the model can provide an adequate insight into trends of virus spread and immunization in the milking group.

The predicted detection time was very similar to the observed value. The discrepancy between the model prediction and data (e.g. ±45 days) may be explained by the fact that the BTM was sampled in different months, while estimates from the model were reported on weekly basis. Therefore, a perfect match between the model output and field data can be difficult to achieve. Moreover, according to Fig. 3, the increasing BTM values seem to relate well to the predicted prevalence of antibody positive milking cows.

Considering the number of dead born calves registered by the farmer, the model predicted few dead born calves. On the other hand, in the field, it is unknown how many

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

calves were born dead due to the BVDV and how many could be born dead due to the interaction of BVDV with other health problems.

4.2. Detection

The threshold prevalence used for each ELISA was based on a pilot study that we carried out for the Danish blocking ELISA and on previously published studies for the SVANOVIR (Niskanen, 1993) and for the Pourquier ELISA (Beaudeau et al., 2001). Those thresholds were the main reason for the difference in the predicted detection time between the three tests. It appeared that the SVANOVIR® BVDV-Ab ELISA is significantly faster at detecting antibodies in BTM than the other ELISAs.

The different detection times of the three ELISAs should be highlighted, because the risk of spreading BVDV to other herds through trading of animals (PIs and TIs) increases with increasing time to detection and is especially relevant when viewed in the light of the frequent movements of animals between Danish herds (Mweu et al., 2013). In Denmark, replacing the Danish blocking ELISA with the SVANOVIR ELISA could allow having a lower disease impact (fewer dead born calves) before detection (Table 5). The risk of spreading the virus to other herds due to animal movements could be limited as well. Further studies investigating the risk of spreading BVDV between herds (before detection) are suggested.

The difference between detection times observed when a PI or a TI was introduced (Table 4), were due to the fact that when TIs are introduced, the amount of virus shed is low and the outbreak can go undetected for long time, especially in large herds (Moerman et al., 1993).

4.3. Impact of model assumptions

Research has shown that airborne spread of BVDV may occur within an infected herd (Bitsch and Rønsholt, 1995; Mars et al., 1999; Bitsch et al., 2000; Niskanen and Lindberg, 2003), and thus this infection route must be considered when modeling the spread of BVDV within a herd. Moreover, it is known that when PIs are present in a herd, the risk of seroconversion in a six months period could be as high as 97% (Houe and Meyling, 1991).

In our model, it is assumed that at least once per day, the virus is sent from the group where one or more PI animals are located (k) to the group where other susceptible animals are (j). Furthermore, it assumes that when PIs are present, there will be a sufficient amount of the virus distributed homogeneously within the herd so that the spread of the virus between groups is independent from the number of animals located within group k. Thus, Eq. (1) illustrates the situation where the PI/s and the susceptible present in different groups are gathered into the same group, but with $\beta_{\mathrm{PIk},j} < \beta_{\mathrm{PI}}$ (representing a lower probability of infection compared to a situation where there is a physical direct contact between the animals). This assumption can be accepted for herd A, because all stables where cows and younger animals were kept, where located within a maximum diameter of 50 m. This could have facilitated the airborne spread of the virus between groups and resulted in a large outbreak. The structure of herd A is generally representative of the Danish dairy herds and thus the results can be generalized for the Danish situation.

In a previously published model (Viet et al., 2004; Ezanno et al., 2007; Ezanno et al., 2008), the between-group spread was driven only by contaminated material (Viet et al., 2004; Viet et al., 2007). Furthermore, Viet et al. (2004) assumed only one contact between any two groups per day and that susceptible animals would not be recurrently exposed to infectious materials or objects during the same day. This is not realistic for Denmark, where herds are generally much larger than in France, where the model by Viet et al. (2004) was developed. This means that there will be more workers, movements between groups and tools used in the Danish herds.

Adopting the notation of Viet et al. (2004) ($\beta_{\mathrm{PIk},j} \times I_{\mathrm{PIk}}/N_j \times N_k$), the parameter $\beta_{\mathrm{PIk},j}$ would correspond to the rate of an infectious transmission from k to j. For large herds (as herd A), this is much different from the rate of an infectious transmission from individuals in k to all of j, which is the parameterization that we use. With parameter values at the level of those used by Viet et al. (2004), the probability of BVDV spreading between groups becomes very low with increasing herd sizes (where $N_j \times N_k$ is large).

For these reasons, we believe that the model by Viet et al. (2004), could be used in countries where the herd size is small (e.g. around 40 cows) (Viet et al., 2004; Ezanno et al., 2007; Ezanno et al., 2008). For Denmark, our model seems to be more applicable, since proper published transmission rates for large herds are lacking.

Regarding the grouping structure of the model, it covers the situation in Denmark. For applications in other countries, additional groups may be relevant. These may be incorporated naturally into the framework of Eq. (1). However, the effects are difficult to predict, because we did not study this in detail (since the grouping structure in Denmark is as we described).

4.4. Sensitivity analysis

The detection time did not change significantly when the within-group transmission rate of PI animals was changed (Table 3, scenario II), because this parameter affected mainly the BVDV spread within the group, in which the PIs were located (mainly young animals) and not the disease dynamics in the milking group. In fact, the probability of antibody detection (Table 3, Z) was similar between scenarios I and II. Those findings are in agreement with findings by Ezanno et al. (2007), who argued that such transmission rate is not a key parameter for predicting BVDV spread in a dairy herd.

Regarding the between-group transmission rate of PI animals (Table 3, scenario III and IV) and the within group transmission rate of TI animals (Table 3, scenario V and VI) it appeared that, as suggested by Ezanno et al. (2007), those are the transmission parameters with the greatest impact on the model outputs, because the spread of virus in the milking group is highly affected. For example, when the transmission rates ($\beta_{\mathrm{PIk},j}$ and β_{TI}) were reduced, the detection time was significantly longer than in the

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

baseline scenario and the probability of detection decreased (Table 3, *Z*), because less milking cows became infected and seroconverted during the simulated period.

When the model was run for less than two years (scenario VII), the detection time was significantly affected, showing that the investigated surveillance period and the BTM sampling frequency, can affect the probability of antibody detection and the detection time. In fact the probability of detection was by far lower in scenario VII than in scenario I and VIII (Table 3, *Z*).

Considering the threshold prevalence, it seems that 50% is adequate for the Danish blocking ELISA, because if 30% was used (scenario IX) detection should have occurred earlier than November 2011, when the BTM was found positive in herd A. The probability of detection was slightly higher for scenario IX than for scenario I, because of the lower prevalence of seroconverted cows needed to have antibody detection (Table 3).

According to the sensitivity analysis it can be said that, scenario I (Table 3) was confirmed to be the closest to reality (data from herd A) and its transmission rates appeared to be the most appropriate to simulate BVDV spread within Danish dairy herds.

4.5. Limitations of the study

Considering the data from herd A that was used to validate the model, it could be argued that not all the calves that were considered as PIs were actually true PIs, because they were not all tested twice three weeks apart. Nonetheless, in the Danish surveillance system, when a herd is classified as BVDV infected, a veterinarian in charge of the control measures must sample all newborn calves at least once per week. If a calf is not PI, but is transiently infected after birth, it needs at least 4–7 days to develop viremia (Brownlie et al., 1987; Baker, 1990) and to have a positive result in the antigen ELISA or in the PCR. Indeed, to have a false-PI calf in the data, (a) it should have been infected at least four days after the veterinarian's previous visit, (b) the vet should have passed only once per week and (c) the calf should not have been tested with a second confirmatory test. In our opinion, the probability of this series of events occurring was low and should not have had a significant impact on our external validation process. Moreover, if a latent period of 4 days is assumed and if the veterinarian visited herd A only once per week (worst case scenario), TI calves could have been sampled only during a short time window (in 3 out of 7 days). In that case, around 60% of the calves reported in Fig. 1 (red line) would have been true PIs, because the sensitivity and specificity of the tests used were close to 100%. The consequence would be that the model could slightly overestimate the number of born PIs. Nevertheless, the Danish Cattle Federation and the personnel working in the laboratories where the samples were tested (Lindholm, DK), confirmed that between 80 and 100% of the calves were actually true PIs.

5. Conclusion

From a general point of view, it can be concluded that the larger the herd size is, the significantly longer the detection time will be, although it must be kept into account, that the route of BVDV introduction (PI vs. TI animals) can play a significant role. The SVANOVIR ELISA showed a significantly shorter time to antibody detection compared to the Danish blocking ELISA. These findings should be considered when placing early warning systems based on bulk milk testing in large herds. The results of the current study provide an important contribution toward optimizing the current surveillance system for BVD in Denmark. The proposed model is a valid tool to simulate BVDV spread within a dairy herd, under herd structures similar to the Danish situation.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was supported by the Ministry of Food, Agriculture and Fisheries of Denmark (Grant number 3412-09-02603) and by the Danish Cattle Federation. The authors are grateful to Dr. Peter Lind, for his critical reading of the manuscript and to Dr. Erik Rattenborg and Prof. Åse Uttenthal for giving us their expert opinion.

References

Ames, T.R., 1986. The causative agent of BVD: its epidemiology and pathogenesis. Vet. Med. 81, 848–869.
Baker, J.C., 1990. Clinical aspects of bovine virus diarrhoea virus infection. Rev. Sci. Tech. Off. Int. Epiz. 9, 25–41.
Beaudeau, F., Belloc, C., Seegers, H., Assié, S., Pourquier, P., Joly, A., 2001. Informative value of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of bovine viral diarrhoea virus (BVDV) antibodies in milk. J. Vet. Med. B 48, 705–712.
Bitsch, V., Rønsholt, L., 1995. Control of bovine viral diarrhea virus infection without vaccines. Vet. Clin. North Am. Food Anim. Pract. 11, 627–640.
Bitsch, V., Houe, H., Nylin, B., Rønsholt, L., 1997. Examination of blood and bulk tank milk samples to monitor the bovine virus diarrhoea infection status of cattle herds In: Edwards, S., Paton, D.J., Wensvoort, G., (Eds.), Proc. 3rd ESVV Symp. Pestivirus Infections, Lelystad, The Netherlands, 19–20 September 1996, pp. 158–161.
Bitsch, V., Hansen, K.-E.L., Rønsholt, L., 2000. Experiences from the Danish programme for eradication of bovine virus diarrhoea (BVD) 1994–1998 with special reference to legislation and causes of infection. Vet. Microbiol. 77, 137–143.
Brownlie, J., Clarke, M.C., Howard, C.J., Pocock, D.H., 1987. Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann. Rech. Vét. 18, 157–166.
Cherry, B.R., Reeves, M.J., Smith, G., 1998. Evaluation of bovine viral diarrhea virus control using a mathematical model of infection dynamics. Prev. Vet. Med. 33, 91–108.
Coria, M.F., McClurkin, A.W., 1978. Duration of active and colostrum-derived passive antibodies to bovine viral diarrhea virus in calves. Can. J. Comp. Med. 42, 239–243.
Council Directive 97/2/EC of 20 January 1997 amending Directive 91/629/EEC laying down minimum standards for the protection of calves.
Courcoul, A., Ezanno, P., 2010. Modelling the spread of bovine viral diarrhoea virus (BVDV) in a managed metapopulation of cattle herds. Vet. Microbiol. 142, 119–128.
Ezanno, P., Fourichon, C., Viet, A.-F., Seegers, H., 2007. Sensitivity analysis to identify key-parameters in modelling the spread of bovine viral diarrhoea virus in a dairy herd. Prev. Vet. Med. 80, 49–64.
Ezanno, P., Fourichon, C., Seegers, H., 2008. Influence of herd structure and type of virus introduction on the spread of bovine viral

diarrhoea virus (BVDV) within a dairy herd. Vet. Res. 39, 1–12, http://dx.doi.org/10.1051/vetres:2008016.
Fray, M.D., Paton, D.J., Alenius, S., 2000. The effects of bovine viral diarrhoea virus on cattle reproduction in relation to disease control. Anim. Reprod. Sci. 60–61, 615–627.
Graat, E.A.M., de Jong, M.C.M., Frankena, K., Franken, P., 2001. Modelling the effect of surveillance programmes on spread of bovine herpesvirus 1 between certified cattle herds. Vet. Microbiol. 79, 193–208.
Halasa, T., Nielen, M., Huirne, R.B.M., Hogeveen, H., 2009. Stochastic bio-economic model of bovine intramammary infection. Livest. Sci. 124, 295–305.
Hartley, P.E., Richards, M.S., 1988. A study of the transmission of bovine virus diarrhoea virus between and within cattle herds. In: Proceedings of the 5th International Symposium on Veterinary Epidemiology and Economics, 1988, Acta Vet. Scand. Suppl. 84, Available at www.sciquest.org.nz
Houe, H., 1993. Survivorship of animals persistently infected with bovine virus diarrhoea virus (BVDV). Prev. Vet. Med. 15, 275–283.
Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet. Microbiol. 64, 89–107.
Houe, H., Meyling, A., 1991. Prevalence of bovine virus diarrhoea (BVD) in 19 Danish dairy herds and estimation of incidence of infection in early pregnancy. Prev. Vet. Med. 11, 9–16.
Houe, H., Baker, J.C., Maes, R.K., Ruegg, P.L., Lloyd, J.W., 1995. Application of antibody titers against bovine viral diarrhea virus (BVDV) as a measure to detect herds with cattle persistently infected with BVDV. J. Vet. Diagn. Invest. 7, 327–332.
Houe, H., Lindberg, A., Moennig, V., 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. J. Vet. Diagn. Invest. 18, 427–436.
Innocent, G., Morrison, I., Brownlie, J., Gettinby, G., 1997. A computer simulation of the transmission dynamics and the effects of duration of immunity and survival of persistently infected animals on the spread of bovine viral diarrhoea virus in dairy cattle. Epidemiol. Infect. 119, 91–100.
Internal Quality Report, Lindholm Laboratories, Denmark, 2005. Påvisning af bovin virus diarreí virus ved TaqMan PCR, pp. 1–8 (in Danish).
Juntti, N., Larsson, B., Fossum, C., 1987. The use of monoclonal antibodies in enzyme linked immunosorbent assays for detection of antibodies to bovine viral diarrhoea virus. J. Vet. Med. B 34, 356–363.
Kendrick, J.W., Franti, C.E., 1974. Bovine viral diarrhea: decay of colostrum-conferred antibody in the calf. Am. J. Vet. Res. 35, 589–591.
Kramps, J.A., van Maanen, C., van de Wetering, G., Stienstra, G., Quak, S., Brinkhof, J., Rønsholt, L., Nylin, B., 1999. A simple, rapid and reliable enzyme-linked immunosorbent assay for the detection of bovine virus diarrhoea virus (BVDV) specific antibodies in cattle serum, plasma and bulk milk. Vet. Microbiol. 64, 135–144.
Kristensen, T., Kristensen, E.S., 1998. Analysis and simulation modelling of the production in Danish organic and conventional dairy herds. Livest. Prod. Sci. 54, 55–65.
Lindberg, A.L.E., Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet. Microbiol. 64, 197–222.
Mars, M.H., Bruschke, C.J.M., van Oirschot, J.T., 1999. Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions. Vet. Microbiol. 66, 197–207.
Moen, A., Sol, J., Sampimon, O., 2005. Indication of transmission of BVDV in the absence of persistently infected (PI) animals. Prev. Vet. Med. 72, 93–98.
Moerman, A., Straver, P.J., de Jong, M.C.M., Quak, J., Baanvinger, T., van Oirschot, J.T., 1993. A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. Vet. Rec. 132, 622–626.
Mweu, M.M., Fournieí, G., Halasa, T., Toft, N., Nielsen, S.S., 2013. Temporal characterisation of the network of Danish cattle movements and its implication for disease control: 2000–2009. Prev. Vet. Med. 110, 379–387.
Niskanen, R., 1993. Relationship between the levels of antibodies to bovine viral diarrhoea virus in bulk tank milk and the prevalence of cows exposed to the virus. Vet. Rec. 133, 341–344.
Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. Vet. J. 165, 125–130.
Niskanen, R., Lindberg, A., Larsson, B., Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Vet. Scand. 41, 93–99.
Pellerin, C., Van Den Hurk, J., Lecomte, J., Tijssen, P., 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260–268.
R Core Team, 2012. R: A Language and Environment For Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/
Rasmussen, T.B., Uttenthal, Å., Reimann, I., Nielsen, J., Depner, K., Beer, M., 2007. Virulence, immunogenicity and vaccine properties of a novel chimeric pestivirus. J. Gen. Virol. 88, 481–486.
Ridpath, J.F., Bolin, S.R., Dubovi, E.J., 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205, 66–74.
Rønsholt L., Nylin, B., and Bitsch V., 1997. A BVDV antigen- and blocking antibody ELISA (DVIV) system used in a Danish voluntary eradication program. In: Edwards, S., Paton, D.J., Wensvoort, G. (Eds.), Proceedings of the 3rd ESVV Symposium on Pestivirus Infections, Lelystad, The Netherlands, Published by the Central Veterinary Laboratory, Weybridge, 150–153.
Sørensen, J.T., Enevoldsen, C., Houe, H., 1995. A stochastic model for simulation of the economic consequences of bovine virus diarrhoea virus infection in a dairy herd. Prev. Vet. Med. 23, 215–227.
Uttenthal, Å., Stadejek, T., Nylin, B., 2005. Genetic diversity of bovine viral diarrhoea viruses (BVDV) in Denmark during a 10-year eradication period. APMIS 113, 536–541.
Viet, A.-F., Fourichon, C., Seegers, H., Jacob, C., Guihenneuc-Jouyaux, C., 2004. A model of the spread of the bovine viral-diarrhoea virus within a dairy herd. Prev. Vet. Med. 63, 211–236.
Viet, A.-F., Fourichon, C., Seegers, H., 2007. Review and critical discussion of assumptions and modelling options to study the spread of the bovine viral diarrhoea virus (BVDV) within a cattle herd. Epidemiol. Infect. 135, 706–721.
Vilček, Š., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M.T., Palfi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. Virol. 146, 99–115.
Zimmer, G., Schoustra, W., Graat, E.A.M., 2002. Predictive values of serum and bulk milk sampling for the presence of persistently infected BVDV carriers in dairy herds. Res. Vet. Sci. 72, 75–82.

Please cite this article in press as: Foddai, A., et al., Stochastic simulation modeling to determine time to detect Bovine Viral Diarrhea antibodies in bulk tank milk. PREVET (2014), http://dx.doi.org/10.1016/j.prevetmed.2014.07.007

Manuscript III

Preventive Veterinary Medicine 116 (2014) 75–88

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Quantitative assessment of the risk of introduction of bovine viral diarrhea virus in Danish dairy herds

Alessandro Foddai[a,*], Anette Boklund[a], Anders Stockmarr[b], Kaspar Krogh[c], Claes Enøe[a]

[a] Section for Epidemiology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark
[b] Statistics and Data Analysis Section, Department of Applied Mathematics and Computer Science, Technical University of Denmark, Matematiktorvet, DK-2800 Lyngby, Denmark
[c] Knowledge Centre for Agriculture, Cattle, Danish Cattle Federation, Agro Food Park 15, Skejby, 8200 Aarhus N, Denmark

ARTICLE INFO

Article history:
Received 27 September 2013
Received in revised form 2 April 2014
Accepted 17 May 2014

Keywords:
BVDV
Risk assessment
Scenario tree model
Stochastic simulations

ABSTRACT

A quantitative risk assessment was carried out to estimate the likelihood of introducing bovine viral diarrhea virus (BVDV) in Danish dairy herds per year and per trimester, respectively. The present study gives important information on the impact of risk mitigation measures and sources of uncertainty due to lack of data. As suggested in the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), the OIE Terrestrial Animal Health Code was followed for a transparent science-based risk assessment. Data from 2010 on imports of live cattle, semen, and embryos, exports of live cattle, as well as use of vaccines were analyzed. Information regarding the application of biosecurity measures, by veterinarians and hoof trimmers practicing in Denmark and in other countries, was obtained by contacting several stakeholders, public institutions and experts. Stochastic scenario trees were made to evaluate the importance of the various BVDV introduction routes. With the current surveillance system, the risk of BVDV introduction was estimated to one or more introductions within a median of nine years (3–59). However, if all imported animals were tested and hoof trimmers always disinfected the tools used abroad, the risk could be reduced to one or more introductions within 33 years (8–200). Results of this study can be used to improve measures of BVD surveillance and prophylaxis in Danish dairy herds.

1. Introduction

Bovine viral diarrhea (BVD) is a disease of domestic and wild ruminants (Olafson et al., 1946; OIE, 2004). It has been eradicated in Denmark, Sweden, Norway and Finland without the use of vaccination (Bitsch and Rønsholt, 1995). In Lower Austria and Switzerland, eradication programs have been launched leading to a significantly reduced prevalence of infected herds (Rossmanith et al., 2010; Presi et al., 2011). Nevertheless, BVD is considered to be distributed worldwide (OIE, 2004) and although its course is usually subclinical, outbreaks can have an important impact on animal health, welfare and economic income for farmers (Sørensen et al., 1995).

BVD is caused by a single-stranded RNA Pestivirus of the Flaviviridae family, which is closely related to Classical Swine Fever (CSFv) and Border Disease viruses (BDv)

* Corresponding author. Tel.: +45 27499397; fax: +45 35886001.
E-mail addresses: alefo@vet.dtu.dk, alessandrotula@hotmail.it (A. Foddai).

http://dx.doi.org/10.1016/j.prevetmed.2014.05.005
0167-5877/

(Collett et al., 1988; Peterhans et al., 2010). Two BVDV species are well described, BVDV-1 and BVDV-2. Recently, discussion arose over the emergence of a new BVDV species (BVDV-3, atypical or 'HoBi'-like bovine Pestiviruses) (Ståhl et al., 2007; Liu et al., 2009). Virus isolates within these groups show biological and antigenic diversity. Moreover, BVDV can be classified in two biotypes: cytopathic and non-cytopathic, according to the damage caused in cell cultures (Corapi et al., 1988; Peterhans et al., 2010).

The principal sources of infection are the persistently infected (PI) animals (Niskanen et al., 2000). PIs have been exposed to BVDV in the uterus before the 120th day of the dam' pregnancy (Brownlie et al., 1987), and will shed the virus in large amounts throughout their lives. Other acutely infected cattle seroconvert 2–3 weeks after infection and obtain lifelong immunity (Baker, 1990). These transiently infected (TI) animals are considered to be of minor importance for the spread of the disease (Niskanen et al., 2000). However, BVDV can circulate within a herd for long time due to TI animals and in the absence of PIs (Moerman et al., 1993).

In Denmark, BVD is considered an exotic disease (Uttenthal et al., 2005). During the study period, all dairy herds were screened quarterly by bulk tank milk (BTM) testing, while beef herds were screened by blood sampling at slaughter.[1] Moreover, milk-producing herds are screened every month (in the BTM) for a six month period, if they have imported animals from other countries. An enzyme-linked immunosorbent assay (ELISA) is used, to detect antibodies against BVDV (Rønsholt et al., 1997) in milk and blood samples. If antibodies are found, the herd is classified as "suspected of harboring PIs", all animals in the herd are tested for BVDV and PIs are eliminated as soon as possible. From 2007 to 2011, three Danish dairy herds out of approximately 4000 were tested positive with BVD. In one herd infected in 2010, BVD was imported with pregnant cows carrying PI calves. In the other two herds, the path of disease introduction is uncertain.

The main routes of BVDV introduction in free countries are considered to be the import of infected cattle or pregnant cows carrying PI calves, contaminated semen, and embryos (Lindberg et al., 2006). Semen and embryos are treated prophylactically during the preparation procedures. However, it cannot be excluded that virus shedding bulls are present in artificial insemination (AI) centers (Polak and Zmudzinski, 1999).

Applying washing and trypsin treatments, as recommended by the International Embryo Transfer Society (IETS), cannot assure complete removal of BVDV from contaminated embryos and ova (Bielanski and Jordan, 1996; Trachte et al., 1998; Gard et al., 2009).

BVDV-transmission via live vaccines (Barkema et al., 2001; Antonis et al., 2004), contaminated equipment and medicines has been reported in the literature (Niskanen and Lindberg, 2003; Katholm and Houe, 2006).

Biting flies have been shown capable of carrying BVDV under experimental conditions, but vector-born transmission has not been shown in the field (Lindberg et al., 2006), while airborne transmission could occur at short distances, e.g. 4–40 m (Mars et al., 1999; Bitsch et al., 2000).

The risk of introducing BVDV to previously uninfected herds via wildlife is usually considered to be very low (Lindberg et al., 2006) and none of the wild deer (roe, fallow, sika and red) tested in Danish studies were tested positive with BVDV (Nielsen et al., 2000; Uttenthal et al., 2007 in Danish).

The risk of disease transmission through contact of cattle with other domestic ruminants (e.g. sheep and goats) can be considered low, and the presence of sheep is not expected to compromise the efficacy of BVD eradication programs (Synge et al., 1999; Lindberg et al., 2006). Danish dairy herds are very specialized on milk production and the proportion of dairy farms with both cattle and sheep (or goats) is small. Moreover, very few sheep and goats are imported to Denmark. According to data obtained from the Danish Cattle Federation (2002–2013), the median number of imported sheep and goats per year is 48 (19–131) and 2 (0–287), respectively. Hence, the risk of introducing BVDV into Danish dairy herds due to import of sheep and goats is expected to be very low.

In this analysis, we focused on risk of BVDV introduction in Danish dairy herds. In Denmark, dairy and beef herds can be considered as two different specialized production types, without much contact between them. Most often, if there are contacts between the two productions types, animals are moved from dairy to beef herds (e.g. male calves). Furthermore, Danish beef herds are recognized as free from BVD. The last case of BVD in Danish beef herds was reported in 2010 and was not related to the case reported in the same year in dairy herds.

Danish farmers export animals to other countries. Keeping the herds free from BVD results in higher revenues from exported animals and better animal health and income for farmers, therefore keeping the Danish dairy population free from BVD is highly prioritized.

The first objective of this study was to estimate the risk of introduction of BVDV to Danish dairy herds (with exposure of at least one animal to the virus), including a description of the relative importance of the different introduction pathways. The second objective was to investigate the impact of intervention strategies, such as compulsory testing of imported animals and disinfecting tools used for cross-border hoof trimming and veterinary practices.

2. Materials and methods

2.1. Data collection and analysis

Datasets on imported (a) live cattle, (b) semen, (c) embryos and on (d) exports of live animals were obtained from the Danish Cattle Federation for the year 2010, while data on (e) vaccines used was obtained for the same year from the Danish register on use of veterinary medicines and

[1] From beef herds, 4 animals are tested every year at slaughter. From herds with imported beef cattle, 2 animals are tested every month at slaughter for a 1 year period.

vaccines (Vetstat[2]). Information on (f) hoof trimmers and (g) veterinarians that could visit cattle herds abroad was not available in registers and was therefore obtained through expert opinions and questionnaires.

Datasets "a–e" were analyzed using the freeware R (version 2.13.2, R Development Core Team, 2010). The descriptive statistics were carried out for all Danish dairy herds which delivered milk at least once in 2010. Information on each milk-producing herd was extracted from the Central Husbandry Register (CHR) and used in the analyses. For imports of cattle, semen and embryos; the country of origin, the quantity and the day of import was registered in the CHR. For exports of Danish cattle, information was also available on date and countries of destination. Moreover, we contacted five Danish export companies by phone to gain knowledge on the number of herds visited abroad by trucks each time they cross the border.

No data was available on veterinarians and hoof trimmers crossing borders and treating animals in other countries. Therefore, we created five questionnaires (I–V) in order to estimate the numbers and frequencies of people crossing borders and their usual practices e.g. disinfection of tools used (yes/no) and the number of herds and animals treated in Denmark and abroad. A first questionnaire (I) on both veterinarians and hoof trimmers was sent to 11 experts from the Danish Cattle Federation (*Videncentret For Landbrug*), 15 experts from the Veterinary Flying Squad checking the use of medicines in farms (*Veterinærrejseholdet*), one expert from Vetstat, and four experts from the Danish Veterinary Association (*Den Dansk Dyrlægeforening*). A second questionnaire (II) on hoof trimmers practicing abroad was sent to 14 experts having professional contacts with Danish hoof trimmers. Furthermore, three questionnaires were sent through the Danish Cattle Federation to 35 Danish hoof trimmers (III), 400 Danish vets (IV) and 175 Danish dairy farmers (V), respectively. Finally, questionnaire (IV) was also sent by the Danish Veterinary Association to 250 Danish vets practicing in dairy herds.

Questionnaires I and II were in English and were composed of 38 and 16 questions, respectively. The other questionnaires (III–V) were in Danish. Questionnaires III and IV contained 16 questions, while questionnaire V had 10. All questionnaires were based on closed questions. For instance, we asked to the veterinarians and hoof trimmers how many times per year they can visit cattle herds abroad (minimum, average and maximum number).

2.2. Risk model

The quantitative model was based on multi-level binomial models developed in 2000 by USDA and as in Bronsvoort et al. (2008). The outcome of the model is the probability that BVDV is introduced to a country by different pathways. This probability was calculated per year and per trimester. In the latter case we investigated, how the risk can change between four consecutive BTM surveys in a one-year period.

Information gained by data analysis and questionnaires was fed into stochastic scenario trees. For each introduction route, a scenario tree was created (Fig. 1 and Appendix Fig. A–D) and the risk of introduction for each route and in total was calculated. A herd was considered to become infected if at least one animal of any age was exposed to BVDV and became viremic (virus positive) or if a live infected animal (TI or PI) was imported. Introduction routes for which the risk was considered to be negligible were not included in the model (insects biting, airborne, and contact of cattle with other domestic and wild ruminants). Based on the results from the questionnaires, we considered the risk of BVDV introduction due to veterinarians practicing across borders, and due to use of inactivated vaccines to be very low (see results below), and therefore we did not build the respective scenario trees.

Input parameters for distributions in the scenario trees were based on available data, literature, expert opinion and questionnaires (Tables 1–8). The models were run in an Excel spreadsheet (Microsoft Office Excel, 2007) using the software @Risk 6 (Palisade Corporation). Latin hypercube sampling with 10,000 iterations and random seeds was used. Moreover, the scenario trees for imported live cattle and embryos were made following the examples in the OIE Handbook on Import Risk Analysis for Animals and Animal Products (OIE, 2010).

The impact of two intervention strategies was investigated, (1) making the testing of imported animals compulsory and (2) always disinfecting the tools used for hoof trimming. Altogether, we developed five scenario trees to describe live-animal imports (*PAnim*, Section 2.2.1), semen imports (*PSem*, Section 2.2.2), embryo imports (*PEmb*, Section 2.2.3), truck visits (*PTruck*, Section 2.2.4), and hoof trimmers practicing across borders (*PTrim*, Section 2.2.5).

The prevalence of herds infected with BVDV in other countries (BHP) was used as input in all scenario trees, while the within herd prevalence (WHP) of virus-positive animals was used in all trees apart from the *PTruck* tree. We used a Pert distribution to model the prevalence in endemic (BHPE) and free countries (BHPF), respectively. The minimum and maximum reflected the minimum and maximum prevalences reported in endemic or free countries, while the median of reported prevalences reflected the most likely value. For the WHP we used as maximum the within herd prevalence of viremic animals (PI plus TI) given by Billinis et al. (2005) (Table 1).

In each scenario tree, probabilities were multiplied along limbs. The likelihood of disease arrival for each BVDV introduction pathway was then obtained by $1-(1-P)^N$, where P was the probability of BVDV introduction due to a single imported commodity e.g. one animal, or one dose of semen, etc., and N was the number of imported animals, or doses of semen, etc. If the tree had two ends showing BVDV introduction, P was calculated as the sum of the probabilities of both terminals (e.g. Fig. 1).

[2] Since mid-2000 any use or handing out of prescription (regarding drugs used for food or for producing animals, including medicated feeding stuffs, sera and vaccines), must be recorded by the veterinarian and reported to an official register called Vetstat (http://www.foedevarestyrelsen.dk/english/animal/animalhealth/veterinary_medicine/Pages/default.aspx).

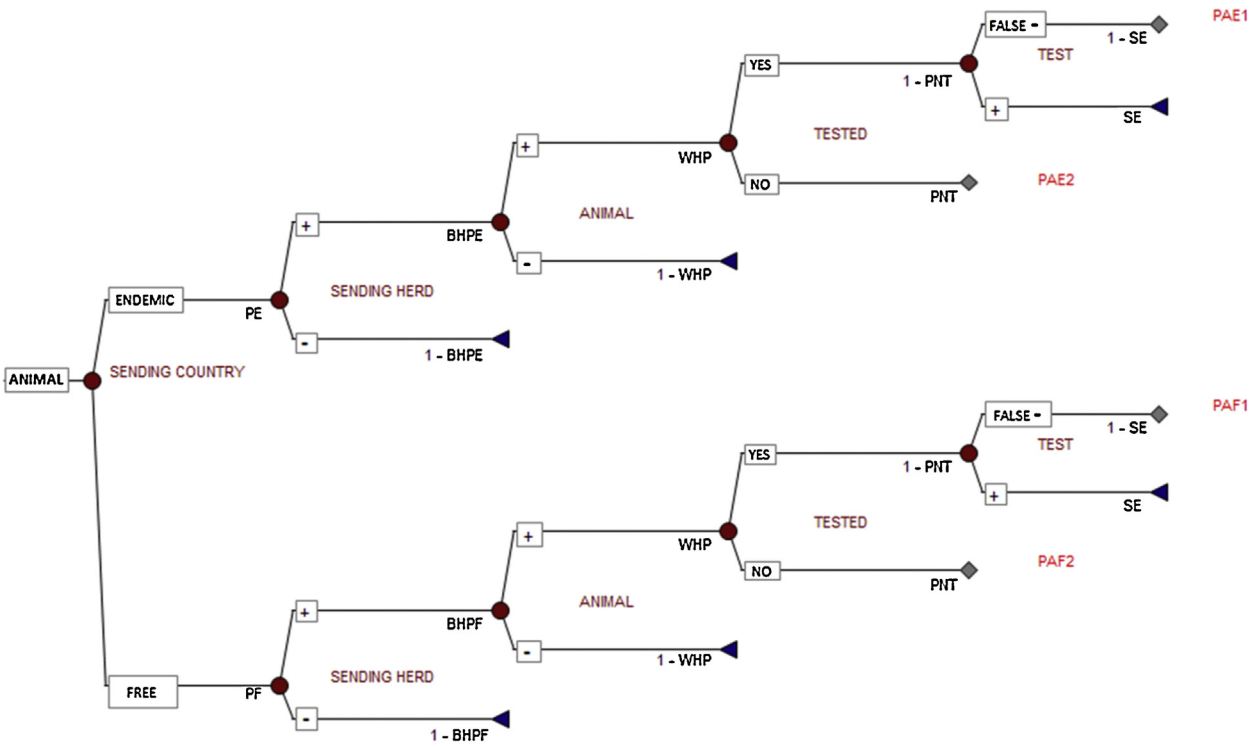


Fig. 1. Scenario tree describing the risk of introducing BVDV from abroad with imported live animals (*PAnim*). PAE1 = probability of introducing an infected tested animal from an endemic country, PAE2 = probability of introducing an infected non-tested animal from an endemic country, PAF1 = probability of introducing an infected tested animal from a free country, PAF2 = probability of introducing an infected non-tested animal from a free country.

The likelihood of BVDV introduction (per year and per trimester) was then estimated by combining information from all scenario trees in the following equation:

$$1 - [(1 - PAnim) * (1 - PSem) * (1 - PEmb) * (1 - PTruck) * (1 - PTrim)] \quad (1)$$

2.2.1. Import of live animals

Cattle are imported to Danish dairy herds from countries with or without endemic BVD (Table 2). Therefore, the *PAnim* tree was divided into two branches, describing these two types of import (Fig. 1). The probability that the animal arrived from an endemic (PE) country (Table 3, Fig. 1) was calculated as beta distributions using the data in Table 2. That beta distribution was obtained by $\alpha = s + 1$ and $\beta = N\text{-}s + 1$, where s is the number of animals coming from the endemic country and N is the total number of animals imported in the year by Danish dairy farmers. The probability that the animal arrived from a free country PF (Table 3, Fig. 1) was calculated as 1-PE. The probabilities that the herd abroad is infected (BHPE, BHPF) and that an

Table 1
Between-herds (BHP) and within-herd prevalence (WHP) reported in the literature in countries with endemic BVD or countries free from BVD.

Endemic Country	BHP	WHP	References
Belgium	RiskUniform (3.80%; 5.00%)	RiskUniform (0.10%; 0.60%)	Sarrazin et al. (2013)
Switzerland	2.00%	0.24%	Presi et al. (2011)
Germany	45.30%[a]	RiskUniform (0.90%; 1.50%[a])	Deregt (2001); [a]Lindberg et al. (2006)
Spain	26.00%	0.70%	Lindberg et al. (2006)
United Kingdom	65.50%[a]	RiskUniform (0.40%[b]; 1.80%[c])	[a]Paton et al. (1998),[b]Deregt (2001), [c]Lindberg et al. (2006)
Ireland	49.60%[a]	0.75%[b]	[a]Lindberg et al. (2006), [b]Stott et al. (2012)
The Netherlands	35.00%	2.22%*	Zimmer et al. (2002)
USA		RiskUniform (0.10%; 1.90%)	Deregt (2001)
Greece		18.00%	Billinis et al. (2005)
Free Country			
Denmark	0.02%[a]	RiskUniform (1.10% [b]; 1.40%[b])	[a]From last outbreak in 2010, 1/4255 herds, [b]Deregt (2001)
Sweden**	0.02%[a]	RiskUniform (1.30% [b]; 1.70%[b])	[a]Ståhl and Alenius (2012), [b]Deregt (2001)

N.B. "RiskUniform" = uniform distribution.

* Given by the average number of PIs found in infected herds/average number of animals in the herd (Zimmer et al., 2002).

** In 2010, Sweden had a similar population to Denmark (approximately 4252 herds) (Alvåsen et al., 2012) and two herds were found positive in 2010 and one in 2011 ([a]Ståhl and Alenius, 2012).

Table 2
Number of animals, semen doses and embryos imported from each country to Danish dairy herds, and destinations of trucks (2010) exporting animals from dairy herds.

Endemic countries	Animals	Semen	Embryos	Trucks destinations
Australia	0	1303	2	0
Austria	0	41	0	0
Belgium	0	1156	1	0
Canada	0	16,391	61	0
Czech Rep.	0	344	0	2
Germany	1	23,125	34	373
France	0	3835	0	4
Great Britain	2	2230	7	1
Hungary	0	1905	0	1
Ireland	0	41	1	0
Italy	0	5368	0	0
Luxemburg	0	186	0	0
Netherlands	23	46,213	77	4758
New Zealand	0	136	0	0
Spain	0	38	0	1
Switzerland	0	216	4	0
USA	0	42,034	88	0
Free countries				
Finland	0	54,397	0	0
Norway	0	27	0	0
Sweden	220	105,810	2	0
Total	246	304,796	277	5140

Table 3
Input used in the scenario tree representing import of live animals (*PAnim*).

Input	Explanation	Values	Sources
PE	Probability the animal is from an endemic country	RiskBeta (26 + 1; 246 – 26 + 1)	Danish data from Table 2
PF	Probability the animal is from a free country	1 – PE	Danish data from Table 2
PNT	Probability the farmer does not test the animal	RiskBeta (1 + 1; 8 – 1 + 1)	Danish Cattle Federation

Table 4
Inputs used in several scenario trees.

Input	Explanation	Values	Sources
BHPE	Between-herd prevalence in endemic countries	RiskPert (2%; 40%; 91%)	Min, median and maximum calculated from Table 1, endemic countries only.
BHPF	Between-herd prevalence in free countries	RiskPert (0; 0.02%; 0.05%)[a]	Min, median and maximum calculated from Table 1, free countries only.
WHP	Within-herd prevalence of viremic animals	RiskPert (0.24%; 1.0%; 18%)	Min, median and maximum calculated from Table 1, endemic and free countries.
1 – SE	Probability of false negative ELISA	1 – RiskPert (90%; 97%; 100%)	Rønsholt et al. (1997)

[a] The maximum represents 2/4255 infected herds in Denmark. Since 2007 maximum 1 dairy herd per year has been classified as BVD positive.

Table 5
Description of inputs used in the semen scenario tree (*PSem*).

Input	Explanation	Values	Sources
PE	Probability the semen is from an endemic country	RiskBeta (144,562 + 1; 304,796 – 144,562 + 1)	Danish data from Table 2
PF	Probability the semen is from a free country	1 – PE	Danish data from Table 2
$(1 - SE)^2$	False negative bull tested twice (ELISA)	[1 – (RiskPert (90%, 97%, 100%))]^2	Rønsholt et al. (1997)
PAIC	Prevalence infected bulls within artificial insemination center	RiskBeta (5 + 1; 219 – 5 + 1);	Polak and Zmudzinski (1999)
		RiskBeta (12 + 1; 1538 – 12 + 1)	Howard et al. (1990)
PVS	Probability a naïve cow gets viremia by 1 dose	RiskBeta (1 + 1; 3 – 1 + 1);	Niskanen et al. (2002)
		RiskBeta (3 + 1; 60 – 3 + 1)	Kirkland et al. (1997)

N.B. In PAIC and PV the two Beta distributions were combined in one input cell by: (a) taking the average (μ) of the means of the two beta distributions and the average variance (δ^2) of the variances of the two beta distributions, and b) setting a final beta distribution with: $\alpha = (\mu^*(\mu^*(1-\mu) - \delta^2)/\delta^2$; $\beta = (1-\mu)^*(\mu^*(1-\mu) - \delta^2)/\delta^2$ as these choices result in a beta distribution with mean and variance equal to μ and δ^2, respectively.

Table 6
Description of inputs used in the embryos scenario tree (*PEmb*).

Inputs	Explanation	Values	Sources
PE	Probability the embryo is from an endemic country	RiskBeta (275 + 1; 277 – 275 + 1)	Danish data from Table 2
PF	Probability the embryo is from a free country	1 – PE	Danish data from Table 2
POD	Probability the infected embryo develops	RiskBeta (336 + 1; 1054 – 336 + 1)	Bielanski and Jordan (1996)
PWR	Probability the BVDV remains on the embryo after wash	RiskBeta (10 + 1; 20 – 10 + 1) and RiskBeta (2 + 1; 9 – 2 + 1)	Bielanski and Jordan (1996) and Trachte et al. (1998)[a]
PVE	Probability a naïve cow gets viremia by 1 infected embryo	100%	Gard et al. (2010)

[a] We considered the probability that the embryo was infected with cytopathic biotype and was still positive to BVDV after washing with trypsin. In PWR the two beta distributions were combined as for PAIC and PV in the *PSem* tree.

imported animal could have BVDV (WHP) are described in Tables 1 and 4.

In Denmark, there is a voluntarily testing program for individual imported cattle. The Danish Cattle Federation recommends that farmers test each imported animal for BVDV antigen (Rønsholt et al., 1997) to detect PI and TI animals, and that imported cattle are kept quarantined until the test results are available. Imported pregnant cows should be kept quarantined until calving and their calf should be tested for antigen at birth. If the calf consumed colostrum, it should be tested by PCR (Rasmussen et al., 2007), which has sensitivity and specificity around 100% (Internal quality report, 2005).

From the outbreak data in 2010, we know that one herd out of eight did not test the animals imported. The probability of not testing animals (PNT) (Table 3, Fig. 1) was therefore modeled as a beta distribution with $\alpha = 1 + 1$ and $\beta = 8 - 1 + 1$. In summary, infected cattle could enter Denmark as a result of not being tested or of testing false-negative (1 – sensitivity of the test used), which leads to the following equation (Tables 3 and 4, Fig. 1):

$$PAnim = 1 - [1 - (PE \times BHPE \times WHP \times PNT + PE \times BHPE \times WHP \times (1 - PNT) \times (1 - SE) + PF \times BHPF \times WHP \times PNT + PF \times BHPF \times WHP \times (1 - PNT) \times (1 - SE))]^N \quad (2)$$

The sensitivity (SE) of the antigen ELISA used in Denmark is 97.9% and the test has been validated with different BVDV strains (Rønsholt et al., 1997). A Pert distribution with minimum 90%, most likely 97% and maximum 100% was therefore used (Table 4) to include the uncertainty related to different BVDV species.

Moreover, as suggested in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2004), diagnostic methods based on bindings of monoclonal

Table 7
Description of inputs used in the trucks scenario tree (*PTruck*).

Inputs	Explanation	Values used	Sources
*n*1	Number of herds visited each time the truck leaves Denmark	RiskPert (1; 3; 4)	The min, most likely and maximum were the median of the estimates given by the 5 exporters
1 – (1 – BHPE)^*n*1	Probability a truck visits an infected herd abroad.	1 – [(1 – RiskPert (2%; 40%; 91%))^*n*1]	Table 1.
PC	Probability the truck is contaminated with BVDV	RiskPert (0.75%; 2%; 5%)	The min, most likely and maximum values were the medians of estimates given by the 3 epidemiologists and 3 virologists
1 – PR	Probability the virus is not removed by the disinfection	1 – [RiskPert (80%; 90%; 100%)]	As for PC
PS	Probability the virus survives after disinfection until Danish herd	RiskPert [(0; RiskPert (0%; 5.9%;13%); RiskPert (28%; 51%; 73%)]	The two RiskPert are medians of averages and 95% confidence intervals given by Stevens (2009), on risk of BVDV survival after 12 and 2 h respectively (on rubber, galvanized metal, enameled metal and soil)
PV	Probability at least one animal has viremia in a Danish herd due to a contaminated truck	RiskPert (0; 0.05%; 0.15%)	As for PC

Table 8
Description of inputs used in the scenario tree of hoof trimmers (*PTrim*).

Input	Explanation	Values used	Sources
$n1$	Hoof trimmers crossing borders in a year	RiskPert (5; 7; 18)	A
$n2$	Times each hoof trimmer crosses the border in a year	RiskPert (1; 8; 30)	A
$n3$	Herds visited abroad per year by all hoof trimmers	RiskPert (1; 10; 50)	A
$n4$	Herds visited abroad each time an hoof trimmer crosses the border	$n3/(n1 \times n2)$	
$1-(1-\text{BHPE})^{n4}$	Prob. a hoof trimmer visits an infected herd abroad	$1-[(1-\text{RiskPert}(2\%; 40\%; 91\%))^{n4}]$	Table 1
$n5$	Animals treated in a visited herd abroad	RiskPert (1; 10; 20)	A
$1-(1-\text{WHP})^{n5}$	Prob. a hoof trimmer treats an infected animal in a herd abroad	$1-[(1-\text{RiskPert}(0.24\%; 1.00\%; 18\%))^{n5}]$	Table 1
PD	Prob. a hoof trimmer disinfects the tools used	RiskPert (0; RiskBeta (2 + 1; 3 − 2 + 1); 100%)	A
PR	Prob. BVDV is completely removed by disinfection	RiskPert (95%; 99%; 100%)	B
PS	Prob. BVDV survives after 12 h	RiskPert (0; 5.9%; 23%)	C
PV	Prob. of viremia in 1 animal treated with disinfected equipment on which BVDV has not been removed	RiskPert (0; 2.7%; 5.8%)	B
PVN	Prob. of viremia in one animal treated with NON disinfected equipment	RiskPert (1%; 45%; 60%)	B
$n6$	Animals treated in a visited Danish herd	RiskPert (1; 120; 500)	A
PVD	Prob. at least one of the animals treated with the disinfected but still contaminated equipment develops viremia in the first visited herd	$[1-(\text{PV})]^{n6}$	
PVND	Prob. at least one of the animals treated with the non disinfected and still contaminated equipment develops viremia in the visited herd	$[1-(\text{PVN})]^{n6}$	
N	Total herds visits at risk in a year	$n1 \times n2$	

N.B. (A) Expert opinion and questionnaires. (B) Medians by expert panel (3 virologists and 3 epidemiologists). (C) The min, most likely and max values are medians of survival probabilities (95%CI: lower limit, average and upper limit) given by Stevens (2009) in a 12 h period (rubber, galvanized metal, enabled metal, and soil).

antibodies (MAb-binding) or on nucleic acid recognition must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses (OIE, 2004, Chapter 2.10.6, Section B.1). If other tests were used abroad, we assumed that they had similar sensitivity, as the ELISA (Rønsholt et al., 1997) and the PCR (Rasmussen et al., 2007), we mentioned.

2.2.2. Import of semen

Semen is also imported to Danish dairy herds from countries with endemic BVD and from countries free from BVD (Table 2). In this scenario tree, the PE and PF were calculated with the same formulas described in Section 2.2.1. Bulls entering artificial insemination (AI) centers must be tested twice for antibodies and for virus (or for antigen) 28 days apart before being accepted (Council Directive, 2003/43/EC). Therefore, the probability of a false negative result from the two testing steps was calculated as $(1-\text{SE})^2$. The SE was set as in the *PAnim* tree. We assumed that both, the antigen and the antibody ELISAs, used on virus positive and antibody positive animals respectively, had sensitivity similar (around 97%) to that of the Danish blocking ELISAs (Rønsholt et al., 1997).

The probability that semen was sampled from an infected bull was set according to the prevalence of infected bulls (PAIC) found in previous studies at infected AI centers (Howard et al., 1990; Polak and Zmudzinski, 1999).

Moreover, semen must be tested for BVD virus (or antigen) if the bull entered the AI center as antibody positive, and bulls must be tested once per year for antibodies, if they entered as antibody negative (Council Directive, 2003/43/EC; Lindberg et al., 2006). In the latter case, if the animal tests antibody positive, semen must be tested for virus or for antigen (Council Directive, 2003/43/EC).

As a conservative scenario, we assumed that all bulls were tested only twice, and if virus was present in the semen of antibody positive bulls, it would be detected due to the high sensitivity of the PCR, which is usually the test of preference for semen.

The probability that a receiving cow would become viremic due to insemination with infected semen (PVS) was based on previous studies (Kirkland et al., 1997; Niskanen et al., 2002). Then, the overall probability of introducing BVDV due to import of semen was calculated as (Table 5, Appendix Fig. A):

$$PSem = 1-[1-(\text{PE}\times\text{BHPE}\times\text{WHP}\times(1-\text{SE})^2\times\text{PAIC} \times\text{PVS}+\text{PF}\times\text{BHPF}\times\text{WHP}\times(1-\text{SE})^2\times\text{PAIC}\times\text{PVS})]^N \quad (3)$$

2.2.3. Import of embryos

Embryos could be a source of BVD infection (Gard et al., 2010). The probability that an infected embryo develops and is ready for implantation (POD), and the probability that BVDV is not removed after the washing procedures (PWR) recommended by the IETS, were calculated according to information from the literature (Table 6, Appendix Fig. B):

$$PEmb = 1-[1-(\text{PE}\times\text{BHPE}\times\text{WHP}\times(1-\text{SE})\times\text{POD} \times\text{PWR}\times\text{PVE}+\text{PF}\times\text{BHPF}\times\text{WHP}\times(1-\text{SE})\times\text{POD} \times\text{PWR}\times\text{PVE})]^N \quad (4)$$

Imported embryos could be produced in vitro (IVP) or could be derived in vivo (IVD). From a general point of view, IVP embryos represent a higher risk of BVDV spreading, than IVD embryos. Unfortunately, it was not possible to get information on the ratio between IVP/IVD among the imported embryos. Therefore, we did not distinguish between IVP and IVD embryos.

Instead, we still considered the probability that the embryo remained positive to BVDV after washing, because embryos could be contaminated by a virus-positive donor that could give positive follicular fluid and recovery medium, which can come in contact with the oocyte/embryo (Bielanski and Jordan, 1996; Stringfellow and Givens, 2000). Both kinds of embryo contaminations, with cytopathic and non-cytopathic biotype (Bielanski and Jordan, 1996; Trachte et al., 1998), were considered in PWR (Table 6, Appendix Fig. B).

Calf sera can be used as culture media for IVP embryos or to collect non-surgically IVD embryos (Waldrop et al., 2004). In previous studies, fetal calf sera batches (FCS) have been shown to be contaminated with BVDV (Bolin et al., 1991; Bolin and Ridpath, 1998). On the other hand new guidelines have been suggested to further reduce this risk. As reported in the Terrestrial Animal Health Code (2007, Article 3.3.1.6), all products of animal origin used in the media and solutions for collection, processing and washing of embryos should be sterilized by methods approved by the IETS Manual. In this study, we therefore decided not to include the risk from fetal calf sera, assuming that only gamma irradiated and heat threated bovine sera was used (Perry, 2007).

In relation to the risk from embryos, semen contamination with BVDV was not considered, because doses used for producing embryos should arrive from AI centers and could be further tested for BVDV (Terrestrial Animal Health Code, 2007, Article 3.3.1.4). We therefore assumed that semen used to produce embryos was free from BVDV, which is in accordance with Perry (2007).

The probability that an infected embryo caused viremia (PVE, Table 6, Appendix Fig. B) in the receiving cow was assumed to be 100%, based on a study by Gard et al. (2010), where infected embryos caused viremia in all (10/10) receiving cows. This represented the worst-case scenario for the imported embryos. The impact of that assumption (PVE = 100%) was investigated in the sensitivity analysis (see Section 2.3).

2.2.4. Trucks visiting Danish dairy herds after being abroad

According to the data analysis and information received by the five transportation companies, we found that all exports from Danish dairy herds go to endemic areas (see results section). Hence, the probability that a truck visited an infected herd abroad was calculated as $(1-(1-\mathrm{BHPE})^{n1})$, where $n1$ represented the number of herds visited abroad each time the truck crossed the Danish border. The probability that the truck was contaminated by visiting an infected herd (PC) and the probability that BVDV was not removed during disinfection at the border $(1-\mathrm{PR})$, were obtained by expert opinion (three virologists and three epidemiologists from different countries were consulted). The probability that the virus survives on metal and other livestock equipment until a Danish dairy herd is visited (PS) was set using estimates by Stevens (2009). The probability that the contaminated truck caused viremia (PV) in at least one animal in the Danish herd visited was based on expert estimates. In this tree, the probability of BVDV introduction was (Table 7, Appendix Fig. C):

$$PTruck = 1-[1-((1-(1-\mathrm{BHPE})^{n1})\times \mathrm{PC}\times(1-\mathrm{PR}) \times \mathrm{PS}\times \mathrm{PV})]^{N} \quad (5)$$

We assumed that all trucks crossing borders were washed and disinfected before visiting a Danish dairy herd, as required for the Danish transport standards (Danish Transport Standard, 2012, version 3.0). We also assumed that the first Danish dairy herd is mostly visited 12 h after the truck had been abroad. This assumption was based on the fact that trucks mostly went to the Netherlands in the investigated period (2010, see results below). At least 5½ h are needed to go from Groningen (Northern Netherlands) to Tinglev (Southern Jutland) (www.viamichelin.com) and the truck needs to pass through the disinfection stations. On the other hand (as explained by the five exporters) in the last three years many exports went to Russia (≥50% of the shipments made by the five consulted companies). Because it takes at least 29 h to drive from Moscow to Tinglev, we considered the 12 h period a fair compromise between shipments to the Netherlands and Russia.

For the trip from Flensburg (Northern Germany) to Tinglev, at least half an hour is needed (www.viamichelin.com). In the latter case, we assumed that at least 2 h passed between leaving the herd abroad, carrying out the truck disinfection procedures and visiting the first Danish dairy herd.

From Stevens (2009), we set the probability for BVDV surviving the trip as a Pert distribution. As the most likely value, we used the median probability of survival in rubber, galvanized metal, enameled metal and soil after a period of 12 h. As the maximum we used the probability of survival after a period of 2 h for the same materials (Stevens, 2009). Since the BVDV could survive for three weeks (Pagnini et al., 1984; Edwards, 2000), as the minimum probability of survival in the Pert distribution we used 0, reflecting a case where a Danish herd was visited three weeks after a truck has been abroad. The number of Danish herds visited within a three-week period by one truck returning from export or import was estimated according to data on animal movements (2010). We assumed that all movements involving Danish dairy herds (92,291 in 2010) were made by the same trucks used abroad. Then, a total of 5606 truck visits at risk was estimated ("N" in Eq. (5)).

2.2.5. Hoof trimmers practicing in Denmark and abroad

According to the questionnaires sent to the different stakeholders and experts, hoof trimmers could visit cattle herds in Germany and the Netherlands. For this reason, we used BHPE in the first node of the stochastic scenario tree (Table 8, Appendix Fig. D). The probability that an infected herd was visited abroad was set as $(1-(1-\mathrm{BHPE})^{n4})$, where $n4$ represented the number of herds visited by one

hoof trimmer abroad, each time he crossed the border. The probability that the tools used became contaminated with BVDV was calculated as $(1-(1-\mathrm{WHP})^{n5})$ where $n5$ represented the number of animals one hoof trimmer can treat in each herd visited abroad. The probability that the hoof trimmer disinfects the tools used (PD), the probability that the virus is removed by disinfection (PR), the probability that BVDV survives until a Danish dairy herd is visited (PS) and the probability that the contaminated tool caused viremia in at least one animal (PVD and PVND) were also taken into account. The overall probability of BVDV introduction due to this tree was given by (Table 8, Appendix Fig. D):

$$PTrim = 1-[1-((1-(1-\mathrm{BHPE})^{n4})\times(1-(1-WHP)^{n5}) \times \mathrm{PD}\times(1-\mathrm{PR})\times \mathrm{PS}\times \mathrm{PVD}+(1-(1-\mathrm{BHPE})^{n4}) \times(1-(1-\mathrm{WHP})^{n5})\times(1-\mathrm{PD})\times \mathrm{PS}\times \mathrm{PVND}]^{N} \quad (6)$$

Usually, when hoof trimmers cross the border they work for the entire day abroad (results from questionnaire II). Therefore, the probability that the virus survived until a Danish herd was visited was set using probability values given by Stevens (2009) for a 12-h period. This assumption implies that the Danish animals are treated on the day after the hoof trimmer has been abroad. After the first Danish herd is visited, we assumed that the virus was completely removed from the equipment used, because several animals can be treated within the first Danish herd visited and the virus should be washed away from the equipment.

2.3. Sensitivity analysis

The sensitivity analysis was made in @Risk using tornado graphs and looking at the regression-mapped values. The amount of change in the output due to a plus 1 standard deviation in each input was investigated. In this way, the impact on the final output caused by each input could be measured and a ranking of the different inputs could be made.

As reference scenario, we used the annual risk estimate obtained with the current Danish situation (without the intervention strategies mentioned in Section 2.2). Then, the impact of the different inputs was also investigated, when the risk mitigation measures were applied.

Additionally, we estimated the risk of introducing BVDV, assuming that three testing steps are carried out on all bulls present within AI centers (two on serum before entering the AI center, and one on semen). For that purpose, one extra node for testing of semen in the *PSem* tree (Appendix Fig. A) was included (between nodes: "semen from positive bull" and "cow viremic"). The probability that a bull tests false negative on semen, was set to 1 − SE. We assumed that the test used on a sample of semen (to detect antigen or BVDV) had similar sensitivity of the tests used on serum. Moreover, we assumed that both antibody positive (Voges et al., 1998) and viremic bulls (TI or PI) (Kirkland et al., 1997; Polak and Zmudzinski, 1999) could shed BVDV in their semen.

Finally, we investigated the impact of using probability of viremia due to contaminated embryos <100% (in PVE, Table 6, Appendix Fig. B). For that purpose, the study by Waldrop et al. (2004) was considered. In that study, IVD embryos were artificially contaminated with a high affinity BVDV strain and were washed or trypsin treated according to the IETS standards. Then, the contaminated embryos were put in contact with cultures of uterine tubal cells (UTC), which represented the uterine environment in vivo. Between 9 and 30% of the UTC cultures were infected (Waldrop et al., 2004). Indeed, in the *PEmb* tree (Table 6, Appendix Fig. B), PVE was set with a uniform distribution ranging from 9 to 30%.

3. Results

3.1. Descriptive statistics

In 2010, 4255 Danish dairy herds delivered milk at least once. Eight of these (0.2%) imported cattle from four countries, and as shown in Table 2, most of the animals came from Sweden, where BVD is considered eradicated. The number of cattle imported by dairy herds (Table 2) corresponded to 57% of the total imported. The remaining 43% went to beef herds and directly to slaughterhouses.

Regarding semen, doses were imported from 20 countries to 3653 (85.9%) Danish dairy herds (Table 2). The proportion of doses imported from endemic and BVDV free countries were 47% and 53%, respectively (Table 2).

Considering embryos, those were imported from 10 countries to 45 (1.1%) dairy herds (Table 2). Most of the embryos (99.3%) arrived from endemic countries (Table 2).

Vaccinations were practiced in 771(18.1%) dairy herds using inactivated vaccines and/or immune serum raised in horses.

Considering exports, the total number of animals exported by dairy herds in 2010 was 17,638. The number of animals moved on the same day from the same herd to the same country was minimum one, maximum 52 with a median of three animals. Most of the moved animals were younger than six months and according to the five exporters, up to 45 heifers could fit within a truck. Thus, assuming that a batch of animals moved on the same day could fit within one truck, we estimated the total number of truck movements (Table 2) occurred from 420 (9.9%) dairy herds. Most of those trucks went to the Netherlands and Germany and none to free countries (Sweden, Norway or Finland) (Table 2).

Regarding the questionnaires, 24 (68.6%) out of the 35 interviewed hoof trimmers answered. Only one of them visited cattle herds abroad. He stated that in a year he would visit maximum one herd, where he can treat up to 20 animals. In Denmark, he could visit 100 dairy herds and in each of them treat minimum one, most often 120 and maximum 310 animals. Additionally, he said he would always disinfect the tools used. Among the 14 experts having contact with hoof trimmers and the 11 experts from the Danish Cattle federation, four and three respectively, answered the questionnaires. The median estimates (with minimum and maximum) given by the seven experts and by the hoof trimmer (Table 8, A) were used in the scenario tree *PTrim*

(Appendix Fig. D). Of the 175 farmers, 157 (89.7%) answered the questionnaire, but none used hoof trimmers from other countries.

None of the four sources we consulted (Danish Cattle Federation, Danish Veterinary Flying Squad, Danish Veterinary Association, and Vetstat) had data on veterinarians crossing borders. Forty-seven (11.8%) out of 400 veterinarians contacted by the Danish Cattle Federation answered the questionnaire. Of these, 13 practiced in cattle herds abroad, but none used the same tools or medicines in Denmark and abroad. Nineteen vets (7.6%) contacted by the Danish Veterinary Association answered the questionnaire. Only three practiced in cattle herds abroad and none used the same tools or medicines in Denmark and in other countries. Of the 157 dairy farmers who answered the questionnaire, two used vets from other countries, but only for advisory purposes.

3.2. Model output

The annual risk of BVDV introduction in Danish dairy herds was 10.7% (5th percentile = 1.7%, 95th percentile = 36.6%). Hence, a median of at least one or more BVDV introductions every nine years (3; 59) could be expected if risk mitigation measures (such compulsory testing of imported animals and disinfection of tools used for hoof trimming) were not applied. The highest risk of disease introduction was related to the import of live cattle with a median of 5.0% (0.7%, 22.6%). The hoof trimmers practicing in Denmark and abroad represented the second most important introduction pathway with a median of 2.4% (0.2%; 15.9%). The median risk of BVDV introduction due to imported semen, embryos and trucks visits was 0.4% (1.54E−02%; 5.3%), 1.0% (0.1%; 5.1%), and 0.04% (3.26E−03%; 0.2%), respectively.

If testing of imported animals was made compulsory, the predicted risk for the *PAnim* tree decreased to 0.8% (0.1%; 4.0%). In the scenario tree used for the hoof trimmers, the predicted risk was reduced to 0.07% (4.37E−03%; 0.5%), if tools were always disinfected after use. With the use of both these mitigating measures, the overall predicted risk per year could be reduced to 2.99% (0.5%; 11.9%). In that case, a median of at least one or more BVDV introductions every 33 years (8; 200) could be expected.

In 2010, the risk of virus introduction per trimester (without control measures) ranged from 1.4% (0.2%; 6.0%) in the fourth trimester to 5.9% (1.0%; 22.0%) in the first trimester (Fig. 2).

3.3. Sensitivity analysis

The input having the highest impact on the overall risk estimate was the prevalence of virus-positive cattle within infected herds (WHP). An increase of one standard deviation in the WHP had an impact between 0% and 7.7% in the final output. The second and third most important inputs were the between-herd prevalence (BHPE) in endemic countries (impact 0; 4.1%), and the probability that the farmer did not test (PNT) the imported animals (0; 3.2%), respectively. The total number of herds hoof trimmers could visit abroad (Table 8, *n*3) and the probability that hoof trimmers disinfected their tools (Table 8, PD), where the most important parameters of those obtained by expert opinion and had a maximum impact of 2.0% and −1.8%, respectively. The overall risk estimate changed by less than 1.5% when any of the other inputs was increased by one standard deviation.

When testing imported cattle was set as compulsory and hoof trimmers always disinfect the tools used, the most important input was still the WHP (impact between 0% and 2.8%), followed by the BHPE (impact between 0% and 1.4%), while all other inputs counted for less than 1.0%.

If all bulls present in AI centers were tested at least twice on serum and once on semen, the overall annual risk (of one or more BVDV introductions) would be slightly lower, compared to when only two tests per bull are made on serum before entering to the AI center. In the former case, the median overall risk would be reduced by 1.0% (0.2%; 1.8%), while the median risk due to import of semen would be 30 times lower.

When the probability of viremia due to infected embryos (PVE, Table 6, Appendix Fig. B) was reduced from 100% to 9–30%, the overall annual risk reduced by 1.2% (0.2%, 1.6%), while the median risk due to import of embryos was 19 times lower compared to the scenario with PVE = 100%.

4. Discussion

4.1. Descriptive statistics

According to the data analysis, semen is the commodity imported by the highest percentage of Danish dairy farmers and in the highest quantity (Table 2). This could be caused by the fact that semen is easier to obtain, to transport and to use than live animals and embryos. Moreover, Danish farmers may be aware of the higher risk related to import of animals.

Regarding exports, the data showed that, although the percentage of dairy herds involved is low, the total number of deliveries could be considered as high and the risk due to this route should be taken into account.

Considering vaccines, few doses were used in a low percentage of dairy herds. Once we verified that no live vaccines were used, we decided that the risk of BVDV introduction due to that pathway could be considered negligible. Moreover, as suggested in international guidelines (OIE, 2004), cell cultures and bovine serum (which could be used to produce vaccines) must be tested and proved free from both BVDV and antibodies. Serum supplements used in media should be sterilized, e.g. by Gamma-irradiation at 25–30 kGy (OIE, 2004, Chapter 2.10.6, Section B.1.a). BVDV outbreaks due to vaccination have been reported abroad, but only due to live vaccines (Barkema et al., 2001; Antonis et al., 2004) and use of immune serum raised in horses should not represent a risk (Brock, 2003). Moreover, vaccines used in Denmark are inactivated with phenol or formaldehyde. These chemicals can inactivate the Classical Swine Fever virus (Edwards, 2000), which has been shown to have resistance similar to BVDV at different temperatures and pH (Depner et al., 1992).

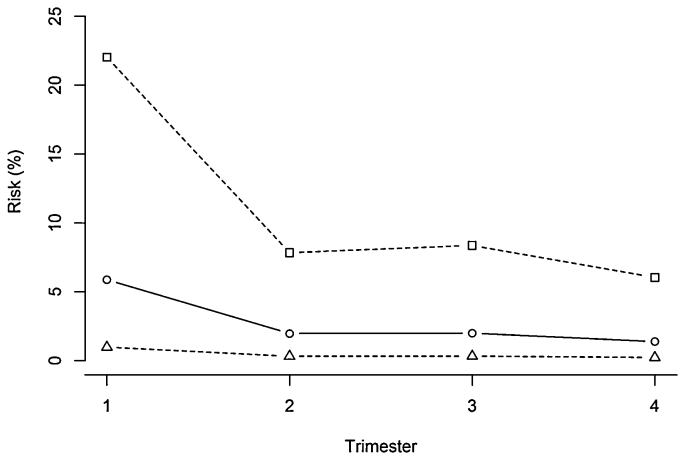


Fig. 2. Risk of BVDV introduction to the Danish dairy population per trimester (1–4) of 2010. ○ = Median risk, △ = 5th percentile, □ = 95th percentile.

Considering the results obtained from questionnaires sent to the different experts and stakeholders, the number of veterinarians and hoof trimmers crossing borders during one year period is very low and veterinarians should not represent a risk.

4.2. Risk estimates

The aim of this study was to investigate the impact of risk mitigation measures and to demonstrate where knowledge needs to be improved so that uncertainty can be reduced. In this way, measures of risk mitigation and BVD surveillance that best meet all of the potential conflicting interests could be set down. We tried to combine the simplicity of the model with a sufficient complexity to represent the different introduction pathways and their importance in a realistic way.

According to our results, individual testing of imported animals could be made compulsory, considering that only few dairy herds and few animals are involved (costs should not be high). Moreover, hoof trimmers should be encouraged to disinfect their tools. With both these simple measures, the risk of BVDV introduction could be reduced markedly.

The scenario trees proposed in this study could be used to identify the herds, with high risk of BVDV introduction during a specific surveillance period (e.g. one year). These herds could be targeted by the surveillance activities. In fact, since dairy herds are surveyed by BTM testing with an antibody ELISA, long time could elapse between BVDV introduction and detection of antibodies in the BTM (due to dilution of individual antibodies in large milk tanks). In herds at higher risk, serum from individuals could be tested instead. The latter approach would be more expensive than testing the BTM, where only one sample is needed. However, the herd sensitivity and the sensitivity of the surveillance system could increase, compared to the surveillance system which is based only on BTM testing.

As shown in Fig. 2, the first trimester of 2010 was the period at higher risk. This finding is in agreement with outbreak data of the same year. In fact, the Danish dairy herd infected in 2010 received cows carrying PI calves in January from a country where BVD is known to be endemic. The receiving Danish herd was classified as BVD infected in November 2010 (287 days after birth of the first PI calf), due to an increase in the BTM antibody titer. The scenario-tree model reflected the change in risk during the investigated periods, by combining information on the quantity of goods imported and their country of origin. If the infected herd had been tested by individual blood sampling after BVDV introduction, it could have been detected earlier.

We are aware that variability between years could be present, because the Danish herd structure is constantly changing. On the other hand, we used data from 2010, which can be considered as a representative year, for the imports of cattle which have been made during the last decade. In fact, between 2002 and 2013, the median number of cattle imported (considering also animals destined to beef herds and to slaughterhouses) was 227 (minimum 74, maximum 1235), which was similar to the number of cattle imported in 2010 (source: Danish Cattle Federation).

Moreover, our model is easy to use and risk estimates could be obtained routinely by feeding into the scenario trees data on imports and exports registered in the central Danish database. If new introductions were proven in the future, the model could be further validated.

Furthermore, the scenario trees proposed in this study could be used for other cattle diseases with similar epidemiology to BVD (e.g. Infectious Bovine Rhinotracheitis, IBR), in which the main sources of infection are the parameters we considered. In the scenario trees of imported live cattle (Fig. 1), semen (Appendix Fig. A) and embryos (Appendix Fig. B), no expert opinion was used. These parameters are considered as the most important means of BVDV spreading between countries (Lindberg et al., 2006). Hence, the impact of eventual bias due to uncertainty related to the expert opinion was limited.

In the trees for trucks (Table 7, Appendix Fig. C) and hoof trimmers (Table 8, Appendix Fig. D) we needed to use expert opinion due to lack of data and literature information. Nevertheless, as suggested by Gustafson et al. (2013), we tried to involve at least five experts (from endemic and free countries) to obtain our estimates, and to include variability between experts.

We invited experts from different organizations and all the main stakeholders (farmers, hoof trimmers, transporters, and veterinarians) of the Danish dairy cattle industry, in order to find their point of view on the risk variables we considered. For instance, the Danish Cattle Federation suggested that we investigated whether veterinarians and hoof trimmers visiting cattle herds abroad could represent a risk factor. According to that suggestion we carried out a deep investigation during a six-month period (117 phone calls and 123 emails). However, the results of the investigation did not indicate that veterinarians used the same tools or medicines in Denmark and abroad, and we therefore excluded veterinarians from our model.

We also investigated, if cattle shows could represent a risk. But we found that in Denmark, international shows are organized only once every two years and no animals are imported from abroad. Similarly, no Danish cattle are sent to other countries for shows (source: Agromek, Viking Genetics and Viking Danmark).

4.3. Limitations of the model

For the within-herd prevalence, we considered as the maximum the prevalence of viremic animals (18%) reported by Billinis et al. (2005) (Table 1). Hence, our estimates could be considered conservative, because lower estimates are usually considered in BVD epidemiological studies (1–2%). These are usually based on prevalence of PIs, which are the main source of infection (Houe, 1999). If only prevalence of PIs had been used as the WHP, risk estimates would have been lower. Moreover, because we were aware that prevalence estimates could change in time and between countries, we tried to consider several epidemiological studies and to combine these in a median estimate. When uncertainty around the estimates was given, this was included (e.g. Table 1, BHP for Belgium). Unfortunately, as highlighted by others (Lindberg et al., 2006), it is rather difficult to find recent prevalence estimates for all the countries considered.

With regard to the vaccines and embryos, it must be said that in cases where international guidelines for sterilizing and testing sera were not fully respected, risk due to both introduction pathways could be higher than we thought and very difficult to estimate. New guidelines have been suggested to further reduce this risk. The Terrestrial Animal Health Code (2007, Article 3.3.1.6), describes that all products of animal origin used in media and solutions for collection, processing and washing of embryos should be sterilized by methods approved by the IETS Manual. Nims et al. (2011) suggested that gamma irradiation at the usual doses may be expected to effectively inactivate any mid-to-large sized viruses (such BVDV) that could be present in the serum. For these reasons, we assumed that the probability of using contaminated FCS batches was 0%, and that only gamma irradiated and heat treated FCS batches could be used, which is consistent with the study by Perry (2007). Moreover, sera batches could be composed of sera collected in different (unknown) areas with different prevalence (free or endemic) and we did not have information regarding the sterilization procedures applied on the FCS batches.

Additionally, we decided to consider all imported embryos as produced in vitro (IVP), because we could not distinguish between IVD and IVP embryos. Usually, it is assumed that it is more difficult to wash adherent BVDV from IVP embryos than from IVD embryos (Bielanski and Jordan, 1996; Trachte et al., 1998; Gard et al., 2009). If some or all the imported embryos were produced in vivo, our results would be an overestimation for the *PEmb* tree. Nevertheless, as shown in Section 3.2, the risk related to this introduction route during a year period is low.

Regarding trucks, we considered that all movements within the country were made with the same trucks used for exports or imports, while in reality at least some of the movements could have been made with trucks owned by the farmer himself, or with other trucks not used for movements abroad. Hence, in reality, the risk of disease introduction due to this path could be lower than the one we estimated. In the following years, uncertainty on the trucks used in Denmark and abroad should be reduced because, at the moment of writing, new legislation is being introduced and the number plate of the truck should be registered by the farmers.

For veterinarians and hoof trimmers practicing abroad, the response rate to our questionnaires could be considered as low. Uncertainty due to lack of knowledge, could be reduced if information on the frequencies they cross the border and their usual practices were registered at the veterinary institutions.

Because only few sheep and goats are imported each year, and usually they are not sent to dairy farms, we assumed the risk from introduction of sheep and goats to be low. If this practice changes over the years, it will need to be taken into consideration in the analyses.

Animal movements from beef to dairy herds should also represent a very low risk, because (a) during the last three years no BVD cases have been reported in the population of beef herds, and (b) usually animals move from dairy to beef herds. Nevertheless, to further reduce the risk of introducing BVDV into Danish dairy herds, we suggest that imported sheep/goats, and beef cattle moved to Danish dairy herds are tested for BVDV or antigen.

4.4. Sensitivity analysis

The high importance of the WHP and the BHPE inputs was expected, because it is logical that the higher the probability an animal/herd is infected, the higher the risk that the virus is sent to Denmark (a viremic animal is imported, etc.). Moreover, both inputs appear in several scenario trees and uncertainty on values reported in the literature was taken into account.

The probability that a hoof trimmer disinfected the tools used and the number of herds visited abroad by hoof trimmers (Table 8, Appendix Fig. D), were the inputs from those obtained from expert opinion, which had the highest importance. On the other hand, the impact on the final outputs was very low.

Adding an antigen test on semen from bull stations to the analysis, had not a relevant effect on the overall annual

risk of BVDV introduction into Danish dairy herds. However, the median risk of introduction for the *PSem* scenario tree was markedly reduced. In countries where more doses of semen are imported (compared to Denmark), the overall annual risk of BVDV introduction could be highly affected. We suggest that semen from all bulls present in AI centers (both antibody positive and not) is tested at least once per year for BVDV or antigen. That could avoid PI bulls, which are not detected in the first two tests, from remaining lifelong within the AI center.

Regarding the embryos, using a high (Gard et al., 2010) vs. a low probability of viremia (Waldrop et al., 2004) in the receiving cow (PVE, Table 6, Appendix Fig. B), only gave a slight effect on the final overall risk of BVDV introduction.

5. Conclusions

According to the present study, the risk of BVDV introduction in Danish dairy herds could be reduced from 10.7% to 2.9% by making testing of imported animals compulsory and disinfecting the tools that hoof trimmers use abroad. Uncertainty on the obtained estimates could be reduced if veterinarians and hoof trimmers practicing across borders were registered. Finally, the present model could be used for other cattle diseases with similar epidemiology to BVD, and to identify the herds with higher risk of BVDV infection during a specific surveillance period.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

Acknowledgements

This study was supported by the Danish Cattle Federation and the Ministry of Food, Agriculture and Fisheries of Denmark (Grant number 3412-09-02603). We are grateful to Prof. Hans Houe, Prof. Åse Uttenthal, Prof. Paul Becher, Dr. Ann Lindberg, Dr. Anette Bøtner, and Dr. Ad Moen who gave us their expert opinion. We thank Dr. Håkan Vigre, Dr. Mariann Chriél and Dr. Lis Alban for giving us their point of view on the assumptions. We are grateful to the Danish Cattle Federation, the Danish Veterinary Flying Squad, the Danish Veterinary Association, the Vetstat, the Agromek, the Viking Genetics and the Viking Danmark for the help and information they gave us during the study. We are grateful the Danish dairy farmers, the hoof trimmers and the veterinarians who kindly answered to our questionnaires.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2014.05.005.

References

Alvåsen, K., Jansson Mörk, M., Hallén Sandgren, C., Thomsen, P.T., Emanuelson, U., 2012. Herd-level risk factors associated with cow mortality in Swedish dairy herds. J. Dairy Sci. 95, 4352–4362, http://dx.doi.org/10.3168/jds.2011-5085.

Antonis, A.F.G., Bouma, A., de Bree, J., de Jong, M.C.M., 2004. Comparison of the sensitivity of in vitro and in vivo tests for detection of the presence of a bovine viral diarrhoea virus type 1 strain. Vet. Microbiol. 102, 131–140.

Baker, J.C., 1990. Clinical aspects of bovine virus diarrhoea virus infection. Rev. Sci. Tech. Off. Int. Epiz. 9, 25–41.

Barkema, H.W., Bartels, C.J., van Wuijckhuise, L., Hesselink, J.W., Holzhauer, M., Weber, M.F., Franken, P., Kock, P.A., Bruschke, C.J., Zimmer, G.M., 2001. Outbreak of bovine virus diarrhea on Dutch dairy farms induced by a bovine herpesvirus 1 marker vaccine contaminated with bovine virus diarrhea virus type 2. Tijdschr. Diergeneeskd. 126, 158–165.

Bielanski, A., Jordan, L., 1996. Washing or washing and trypsin treatment is ineffective for removal of noncytopathic bovine viral diarrhea virus from bovine oocytes or embryos after experimental viral contamination of an in vitro fertilization system. Theriogenology 46, 1467–1476.

Billinis, C., Leontides, L., Amiridis, G.S., Spyrou, V., Kostoulas, P., Sofia, M., 2005. Prevalence of BVDV infection in Greek dairy herds. Prev. Vet. Med. 72, 75–79.

Bitsch, V., Rønsholt, L., 1995. Control of bovine viral diarrhea virus infection without vaccines. Vet. Clin. North Am. Food Anim. Pract. 11, 627–640.

Bitsch, V., Hansen, K.-E.L., Rønsholt, L., 2000. Experiences from the Danish programme for eradication of bovine virus diarrhoea (BVD) 1994–1998 with special reference to legislation and causes of infection. Vet. Microbiol. 77, 137–143.

Bolin, S.R., Matthews, P.J., Ridpath, J.F., 1991. Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. J. Vet. Diagn. Invest. 3, 199–203.

Bolin, S.R., Ridpath, J.F., 1998. Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum. J. Vet. Diagn. Invest. 10, 135–139.

Brock, K.V., 2003. The persistence of bovine viral diarrhea virus. Biologicals 31, 133–135.

Bronsvoort, B.M. de C., Alban, L., Greiner, M., 2008. Quantitative assessment of the likelihood of the introduction of classical swine fever virus into the Danish swine population. Prev. Vet. Med. 85, 226–240.

Brownlie, J., Clarke, M.C., Howard, C.J., Pocock, D.H., 1987. Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann. Rech. Vét. 18, 157–166.

Collett, M.S., Anderson, D.K., Retzel, E., 1988. Comparisons of the pestivirus bovine viral diarrhoea virus with members of the Flaviviridae. J. Gen. Virol. 69, 2637–2643.

Corapi, W.V., Donis, R.O., Dubovi, E.J., 1988. Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections. J. Virol. 62, 2823–2827.

Council Directive, 2003/43/EC of 26 May 2003, 2003. Amending Directive 88/407/EEC laying down the animal health requirements applicable to intra-Community trade in and imports of semen of domestic animals of the bovine species. Off. J. Eur. Union, L 143/23–32.

Danish Transport Standard. Version 3.0, January 2012. http://vsp.lf.dk/danish.aspx

Depner, K., Bauer, T., Liess, B., 1992. Thermal and pH stability of pestiviruses. Rev. Sci. Tech. Off. Int. Epiz. 11, 885–893.

Deregt, D., 2001. Geographical distribution and prevalence of bovine pestivirus infections types I and II. In: Proceedings of the International Workshop Organized by the EDQM on: Pestivirus Contamination of Bovine Sera and Other Bovine Virus Contamination, Council of Europe, Paris, 29–30 March, pp. 25–31.

Edwards, S., 2000. Survival and inactivation of classical swine fever virus. Vet. Microbiol. 73, 175–181.

Gard, J.A., Givens, M.D., Marley, M.S.D., Galik, P.K., Riddell, K.P., Stringfellow, D.A., Zhang, Y., Edmondson, M.A., 2009. Bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced preimplantation bovine embryos following artificial exposure. Theriogenology 71, 1238–1244.

Gard, J.A., Givens, M.D., Marley, M.S.D., Galik, P.K., Riddell, K.P., Edmondson, M.A., Rodning, S.P., 2010. Intrauterine inoculation of seronegative heifers with bovine viral diarrhea virus concurrent with transfer of in vivo-derived bovine embryos. Theriogenology 73, 1009–1017.

Gustafson, L.L., Gustafson, D.H., Antognoli, M.C., Remmenga, M.D., 2013. Integrating expert judgment in veterinary epidemiology: example guidance for disease freedom surveillance. Prev. Vet. Med. 109, 1–9.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet. Microbiol. 64, 89–107.

Howard, T.H., Bean, B., Hillman, R., Monke, D.R., 1990. Surveillance for persistent bovine viral diarrhea virus infection in four artificial insemination centers. J. Am. Vet. Med. Assoc. 196, 1951–1955.

Internal quality report, Lindholm laboratories, Denmark, 2005. Påvisning af bovin virus diarre' virus ved TaqMan PCR., pp. 1–8 (in Danish).

Katholm, J., Houe, H., 2006. Possible spread of bovine viral diarrhoea virus by contaminated medicine. Vet. Rec. 158, 798–799.

Kirkland, P.D., McGowan, M.R., Mackintosh, S.G., Moyle, A., 1997. Insemination of cattle with semen from a bull transiently infected with pestivirus. Vet. Rec. 140, 124–127.

Lindberg, A., Berriatua, E., Fourichon, C., Mintiens, K., Houe, H., 2006. Epidemiology and risks. In: EU Thematic Network on Control of Bovine Viral Diarrhoea Virus (BVDV). BVDV Control QLRT – 2001-01573., pp. 24–72, http://www.afbini.gov.uk/chs-thematic-network-position-paper-on-bvd-control.pdf

Liu, L., Xia, H., Wahlberg, N., Belák, S., Baule, C., 2009. Phylogeny, classification and evolutionary insights into pestiviruses. Virology 385, 351–357.

Mars, M.H., Bruschke, C.J.M., van Oirschot, J.T., 1999. Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions. Vet. Microbiol. 66, 197–207.

Moerman, A., Straver, P.J., de Jong, M.C.M., Quark, J., Baanvinger, T., van Oirschot, J.T., 1993. A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. Vet. Rec. 132, 622–626.

Nielsen, S.S., Roensholt, L., Bitsch, V., 2000. Bovine virus diarrhea in free-living deer from Denmark. J. Wildl. Dis. 36, 584–587.

Nims, R.W., Gauvin, G., Plavsic, M., 2011. Gamma irradiation of animal sera for inactivation of viruses and mollicutes – a review. Biologicals 39, 370–377.

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. Vet. J. 165, 125–130.

Niskanen, R., Lindberg, A., Larsson, B., Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Vet. Scand. 41, 93–99.

Niskanen, R., Alenius, S., Belák, K., Baule, C., Belák, S., Voges, H., Gustafsson, H., 2002. Insemination of susceptible heifers with semen from a non-viremic bull with persistent bovine virus diarrhoea virus infection localized in the testes. Reprod. Dom. Anim. 37, 171–175.

OIE, 2004. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Bovine Viral Diarrhoea Chapter, 2.10.6, http://web.oie.int/fr/normes/mmanual/A_00132.htm

OIE, 2010. Introduction and qualitative risk analysis. Handbook on Import Risk Analysis for Animals and Animals Products, vol. 1., 2nd ed., pp. 32–33.

Olafson, P., MacCallum, A.D., Fox, F.H., 1946. An apparently new transmissible disease of cattle. Cornell Vet. 36, 205–213.

Pagnini, P., Rossi, G., de Tomassis, B., di Matteo, R., Martone, F., 1984. Ricerche sulla resistenza del virus della diarrea virale del bovino agli agenti fisici e nelle acque. Acta Med. Vet. 30, 277–284.

Paton, D.J., Christiansen, K.H., Alenius, S., Cranwell, M.P., Pritchard, G.C., Drew, T.W., 1998. Prevalence of antibodies to bovine virus diarrhoea virus and other viruses in bulk tank milk in England and Wales. Vet. Rec. 142, 385–391.

Perry, G.H., 2007. Risk assessment of transmission of bovine viral diarrhea virus (BVDV) in abattoir-derived in vitro produced embryos. Theriogenology 68, 38–55.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extintion. Vet. Res. 41 (44), 1–14.

Polak, M.P., Zmudzinski, J.F., 1999. Prevalence of bovine viral diarrhea virus infection in bulls in artificial insemination centers in Poland. Vet. Microbiol. 64, 253–257.

Presi, P., Struchen, R., Knight-Jones, T., Scholl, S., Heim, D., 2011. Bovine viral diarrhea (BVD) eradication in Switzerland – experiences of the first two years. Prev. Vet. Med. 99, 112–121.

Rasmussen, T.B., Uttenthal, Å., Reimann, I., Nielsen, J., Depner, K., Beer, M., 2007. Virulence, immunogenicity and vaccine properties of a novel chimeric pestivirus. J. Gen. Virol. 88, 481–486.

Rønsholt, L., Nylin, B., Bitsch, V., 1997. A BVDV antigen- and antibody blocking ELISA (DVIV) system used in a Danish voluntary eradication program. In: 3rd Pestivirus Symposium, Lelystad, Netherlands, pp. 150–153.

Rossmanith, W., Deinhofer, M., Janacek, R., Trampler, R., Wilhelm, E., 2010. Voluntary and compulsory eradication of bovine viral diarrhoea virus in Lower Austria. Vet. Microbiol. 142, 143–149.

Sarrazin, S., Veldhuis, A., Méroc, E., Vangeel, I., Laureyns, J., Dewulf, J., Caij, A.B., Piepers, S., Hooyberghs, J., Ribbens, S., Van Der Stede, Y., 2013. Serological and virological BVDV prevalence and risk factor analysis for herds to be BVDV seropositive in Belgian cattle herds. Prev. Vet. Med. 108, 28–37.

Sørensen, J.T., Enevoldsen, C., Houe, H., 1995. A stochastic model for simulation of the economic consequences of bovine virus diarrhoea virus infection in a dairy herd. Prev. Vet. Med. 23, 215–227.

Ståhl, K., Alenius, S., 2012. BVDV control and eradication in Europe – an update. Jpn. J. Vet. Res. 60, 31–39.

Ståhl, K., Kampa, J., Alenius, S., Persson Wadman, A., Baule, C., Aiumlamai, S., Belák, S., 2007. Natural infection of cattle with an atypical 'HoBi'-like pestivirus – implications for BVD control and for safety of biological products. Vet. Res. 38, 517–523.

Stevens, E.T., 2009. The Persistently Infected Bovine Viral Diarrhea Virus Individual: Prevalence, Viral Survival, and Impact Within Commercial Feeding Systems. Chapter 4., pp. 47–76, http://krex.k-state.edu/dspace/handle/2097/2159

Stott, A.W., Humphry, R.W., Gunn, G.J., Higgins, I., Hennessy, T., O'Flaherty, J., Graham, D.A., 2012. Predicted costs and benefits of eradicating BVDV from Ireland. Ir. Vet. J. 65, 1–11.

Stringfellow, D.A., Givens, M.D., 2000. Preventing disease transmission through the transfer of in-vivo-derived bovine embryos. Livest. Prod. Sci. 62, 237–251.

Synge, B.A., Clark, A.M., Moar, J.A.E., Nicolson, J.T., Nettleton, P.F., Herring, J.A., 1999. The control of bovine virus diarrhoea virus in Shetland. Vet. Microbiol. 64, 223–229.

Trachte, E., Stringfellow, D., Riddell, K., Galik, P., Riddell, M.J., Wright, J., 1998. Washing and trypsin treatment of in vitro derived bovine embryos exposed to bovine viral diarrhea virus. Theriogenology 50, 717–726.

Uttenthal, Å., Stadejek, T., Nylin, B., 2005. Genetic diversity of bovine viral diarrhoea viruses (BVDV) in Denmark during a 10-year eradication period. APMIS 113, 536–541.

Uttenthal, Å., Elbrink, H., Hammer, A.S., 2007. Er rådyr reservoir for Bovin virus diarré virus (BVDV) i Danmark? Rådyr rapport juli 2007. Vetinst AAU, 1–8 (in Danish).

Voges, H., Horner, G.W., Rowe, S., Wellenberg, G.J., 1998. Persistent bovine pestivirus infection localized in the testes of an immune-competent, non-viremic bull. Vet. Microbiol. 61, 165–175.

Waldrop, J.G., Stringfellow, D.A., Galik, P.K., Riddell, K.P., Riddell, M.G., Givens, M.D., Carson, R.L., 2004. Infectivity of bovine viral diarrhea virus associated with in vivo-derived bovine embryos. Theriogenology 62, 387–397.

World Organization for Animal Health, 2007. Terrestrial Animal Health Code, 16th ed., Appendix 3.3.1., pp. 343–344.

Zimmer, G., Schoustra, W., Graat, E.A.M., 2002. Predictive values of serum and bulk milk sampling for the presence of persistently infected BVDV carriers in dairy herds. Res. Vet. Sci. 72, 75–82.

Appendix.

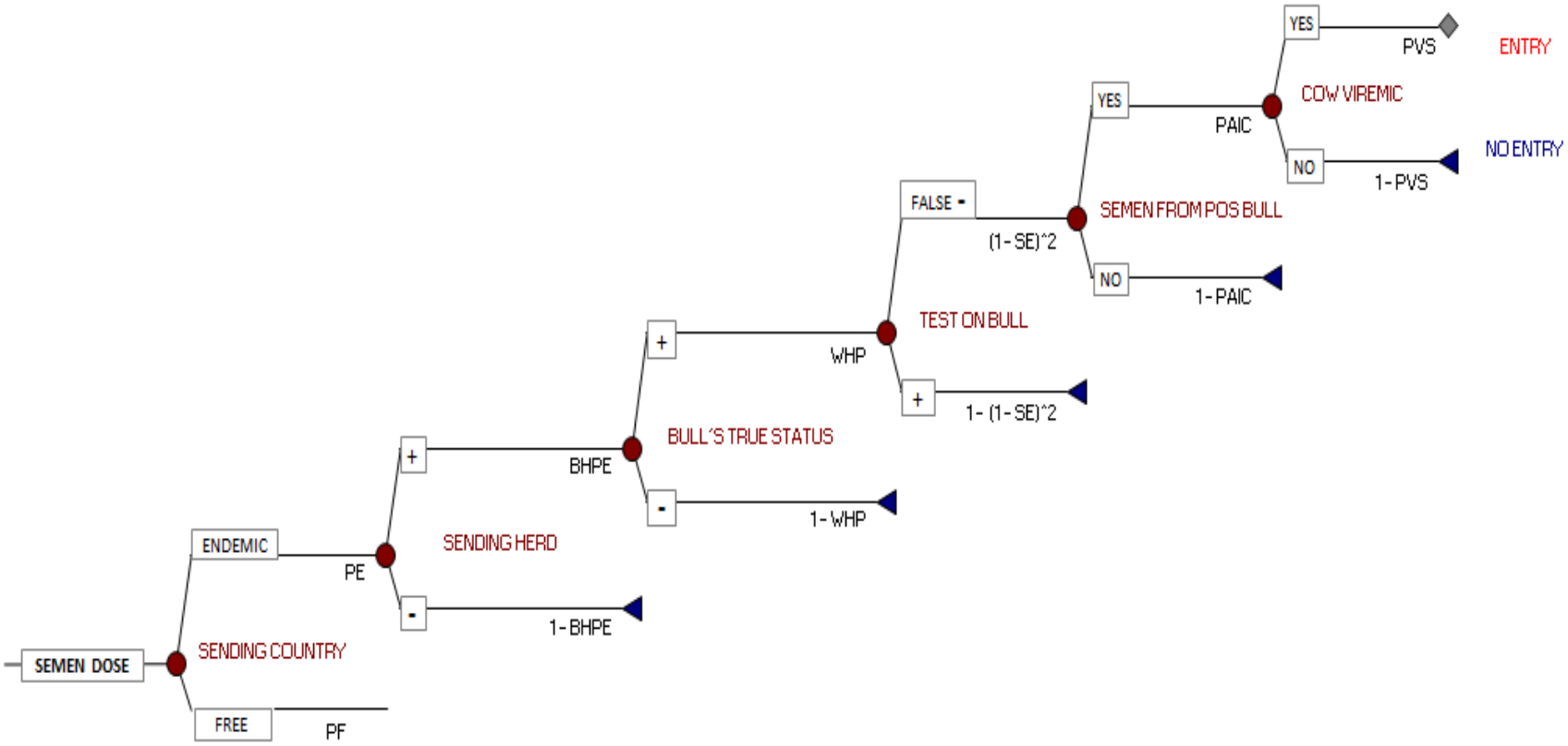


Figure A. Stochastic scenario tree describing the risk of introducing BVDV with imported semen (*PSem).* For free countries a similar branch was made using PF and BHPF

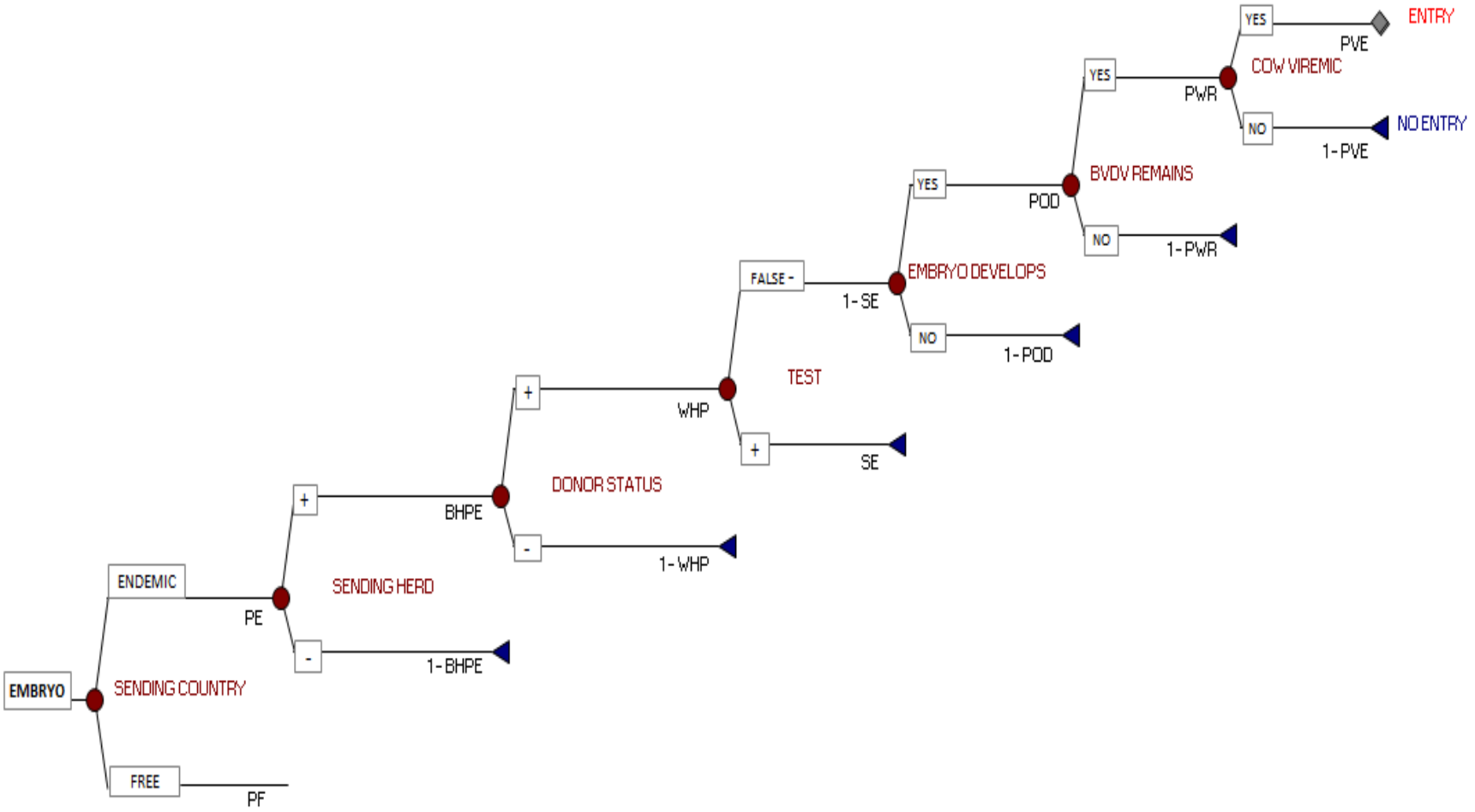


Figure B. Stochastic scenario tree describing the risk of introducing BVDV with imported embryos (*PEmb).* For free countries a similar branch was made using PF and BHPF.

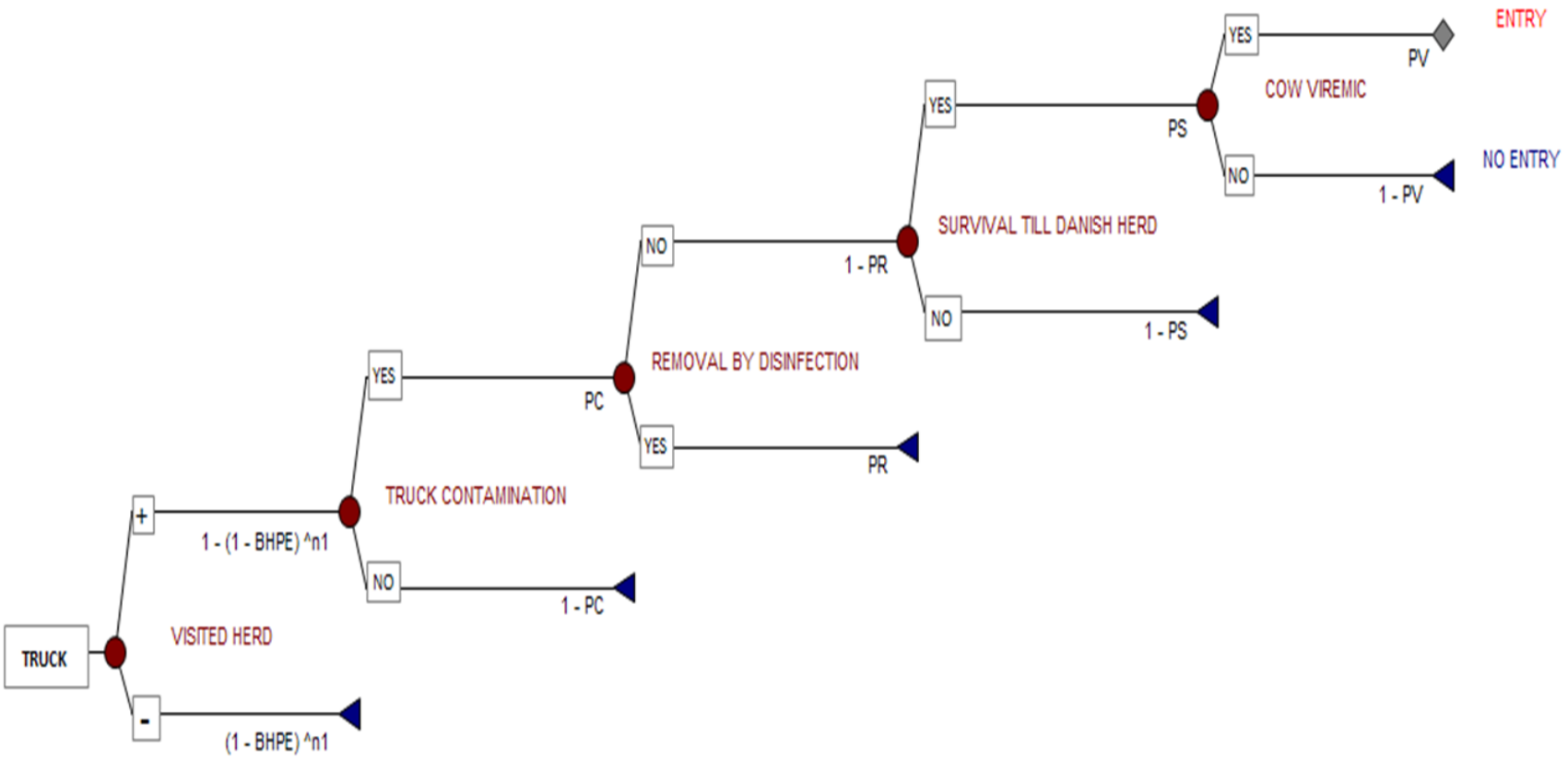


Figure C. Stochastic scenario tree describing the risk of introducing BVDV with trucks visiting Danish dairy herds after export or import (*PTruck)*.

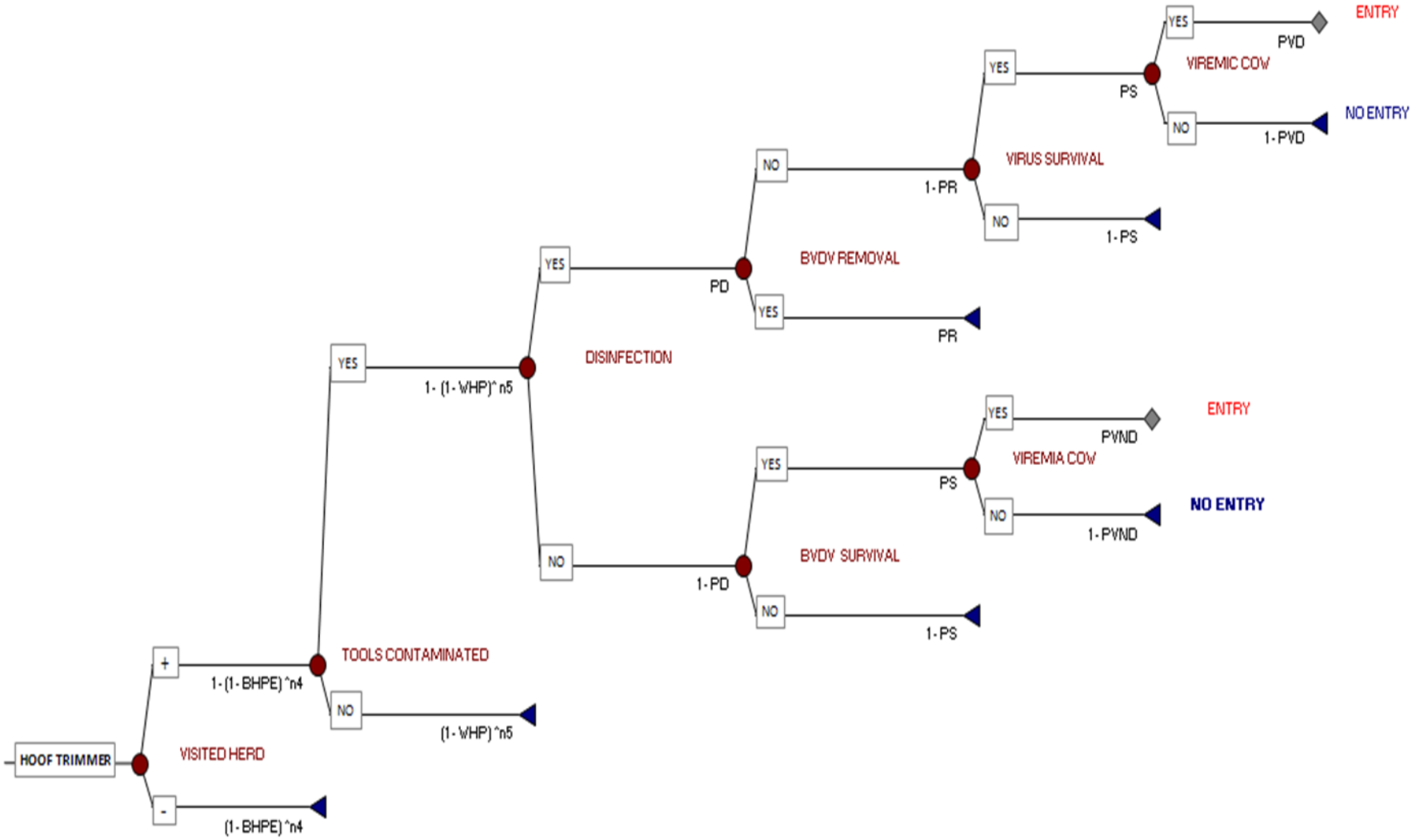


Figure D. Stochastic scenario tree describing the risk of introducing BVDV with hoof trimmers visiting Danish dairy herds after being abroad (*PTrim).*

Manuscript IV

Evaluation and optimization of the temporal surveillance system sensitivity (SSe) for Bovine Viral Diarrhea in Danish dairy herds

Alessandro Foddai[a*], Anders Stockmarr[b], Anette Boklund[a]

[a,]*Section of Epidemiology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark.*

[b,]*Statistics and Data Analysis Section, Department of Applied Mathematics and Computer Science, Technical University of Denmark, Matematiktorvet, DK-2800 Lyngby, Denmark.*

*Corresponding author, Tel. +45 27499397; Fax: +45 35886001

E-mail address: alefo@vet.dtu.dk (Alessandro Foddai)

Abstract

The temporal sensitivity (*SSe*) of the surveillance system for Bovine Viral Diarrhea virus (BVDV) in Danish dairy herds was evaluated. Moreover, measures of *SSe* maximization were investigated. Information from data (2010) and outputs from two stochastic models developed in previous studies was fed into scenario trees. Risk of herd infection, test used and the period from BVDV introduction (into the country) to testing (90 or 365 days) were taken into account. We investigated the effect of introducing one persistently infected calf (PI) or one transiently infected (TI) milking cow, into 1 (or 8) dairy herd(s). Conclusions on the BVD status of the national dairy population were given as: confidence in low (*PLow*) herd prevalence (< 8/4109 infected herds) and confidence in complete freedom (*PFree*) from BVD (< 1/4109). Currently, the Danish blocking ELISA is used to test quarterly bulk tank milk (BTM). As alternative surveillance strategies, we considered (i) using the SVANOVIR ELISA on BTM, and (ii) testing dairy herds at higher risk of BVDV introduction (importing cattle) by individual serum samples and other dairy herds by BTM. From a general point of view, the temporal *SSe*, the *PLow*, and the *PFree* were higher, when tests were performed 365 days after BVDV introduction, than after 90 days. Estimates were usually higher when the SVANOVIR was used, compared to the blocking ELISA, and when a PI rather than a TI was introduced in the herd(s). For instance, with the current system, the median temporal *SSe* was 65% (90% prediction interval: 8%; 97%) 90 days after a PI calf was introduced into at least eight dairy herds. The related *PLow* was 72% (50%; 97%). When a PI calf was introduced into one herd and the same testing strategy was used, the temporal *SSe* was 12% (1%; 36%), while the related *PFree* was 52% (48%; 60%). With the SVANOVIR these parameters were estimated to 99% (82%; 100%); 99% (85%; 100%), 42% (20%; 56%) and 62% (54%; 68%), respectively. Hence, the replacement of the blocking ELISA

with the SVANOVIR could increase the temporal *SSe* and the related *PFree/PLow* remarkably. Testing herds at higher risk of infection in individual serum would not increase the temporal *SSe* noticeably, due to the low number of dairy herds importing cattle. Those results could be used to optimize the surveillance system and to substantiate BVD free status in the Danish dairy population.

Key words: Surveillance system sensitivity; Scenario trees; Freedom from disease.

1. Introduction

Bovine Viral Diarrhea (BVD[1]) is a disease of domestic (Olafson et al., 1946) and wild ruminants, e.g. deer (Haigh et al., 2002), which is caused by a single stranded RNA (+) *Pestivirus* (BVDV) of the *Flaviviridae* family (Collett et al., 1988; Peterhans et al., 2010). Uterine infections in pregnant cows can cause abortions, stillbirths or weak calves (McClurkin et al., 1984; Brownlie et al., 1987; Baker, 1990). Cows exposed to BVDV in the first 120 days of pregnancy, can give birth to calves, which become persistently infected (PI) (McClurkin et al., 1984). PI calves shed the virus in large amounts throughout their lives. Other transiently infected (TI) animals shed the virus in small amounts for 2-3 weeks and become lifelong immune (Brownlie et al., 1987; Baker, 1990).

In dairy cattle herds, BVD surveillance is usually based on testing for antibodies to BVDV in bulk tank milk (BTM) (Niskanen, 1993; Bitsch et al., 1997). The Danish eradication program started in 1994, and during our study period all dairy herds were screened quarterly by bulk milk testing with the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997). Beef herds are screened at abattoir by blood sampling. If a herd is tested positive, individual animals are tested to find at least one antibody positive (sample size determined to have 95% herd sensitivity, assuming 10% within-herd prevalence). If the positive herd status is confirmed, all non-antibody positive animals are tested to detect the viremic animals and PI calves are eliminated.

Between 2007 and 2011, only three Danish dairy herds out of approximately 4100 were diagnosed with BVDV by BTM testing. All three herds had more than 150 cows, and it was

[1] All abbreviations are synthetized in the Appendix

estimated that considerable time elapsed between the day of BVDV introduction and detection of antibodies in the BTM.

Because the Danish dairy herds size increased remarkably since the eradication program started, an evaluation and eventual optimization of the Danish BVD surveillance system was considered necessary by the Danish Cattle Federation. An optimal early-warning system based on BTM testing should detect newly infected herds as soon as possible. Early-warning surveillance systems are those aimed to detect the "unexpected" in a timely way (Hoinville et al., 2013). In Denmark, BVD is considered an exotic disease (Uttenthal et al., 2005), and if it was still present in the country, the prevalence of infected herds should be very low. Hence, detection of newly infected herds can be considered as "unexpected", and an adequate early-warning surveillance system is needed.

An early-warning system should be based on a) the risk that some herd becomes infected and b) the time needed to detect antibodies in BTM. The latter is known to depend on the herd size and on the threshold prevalence of antibody positive milking cows, at which the BTM can be classified as positive with the antibody ELISA used (Graat et al., 2001; Foddai et al., 2014a). The time from a pathogen is introduced into the country, until it is detected by the surveillance activities, has been defined as "high risk period" (*HRP*) (Horst et al., 1997), or "timeliness" (Hoinville et al., 2013). In this study we use the former term, to remark the fact that the longer the time required for detection the higher is the risk the pathogen is spread from the first case(s) herd(s) to other Danish herds.

The probability of detecting a pathogen after a certain time period has been called "temporal sensitivity" (Thurmond, 2003).

The aims of the present study where (i) to evaluate the temporal sensitivity of the Danish BVD surveillance system with fixed *HRPs,* according to different BVDV introduction routes (e.g. import of PI or TI animals), and (ii) to investigate the effect of measures of surveillance optimization[2], by taking into account the risk of BVDV introduction to the country, the herd structure, and the antibody ELISA used.

2. Materials and methods

2.1 Development of the scenario trees

The diagnostic sensitivity of national veterinary surveillance systems (*SSe*) can be evaluated through use of stochastic scenario tree models (Martin et al., 2007a; Martin et al., 2007b; Martin, 2008). Information from different surveillance sources can be combined into an overall *SSe* estimate, taking into account (I) the prevalence of infected herds in a country (between-herds design prevalence, P_H), (II) the prevalence of infected animals within a herd (within-herd design prevalence, P_U), (III) the relative risk (*RR*) of infection in the populations strata, and (IV) the sensitivity (*Se*) of the diagnostic test used (Martin et al., 2007a).

In this study, we defined P_H as 1/4109 (0.02%), reflecting that we aimed at detecting the first infected herd, or as 8/4109 (0.2%) reflecting a low prevalence [(3)], where 4109 was the number of

[2] The term "optimization" is here used to indicate the maximization of the temporal surveillance system sensitivity (*SSe*), with the related confidence in low herd prevalence (*PLow*) and confidence in freedom from BVD (*PFree*).

[3] This design prevalence is set up by World Animal Health Organization (OIE) to substantiate officially free status for enzootic bovine leucosis (EBL) (OIE, 2010 art. 11.9.2), infectious bovine rhinotracheitis (IBR) (OIE, 2010 art. 11.11.2) and bovine tuberculosis (bTB) (OIE, 2013 art. 11.6.2).

Danish dairy herds in October 2010. For BTM testing, P_U was here defined as the threshold prevalence of antibody positive milking cows, at which the BTM was classified positive by the ELISA used, with the assumed test *Se*.

The *SSe* represents the probability that a population, infected with the assumed P_H and P_U, is correctly classified by the surveillance system.

Moreover, usually, in the methodology proposed by Martin et al. (2007a) it is assumed that the surveillance components (e.g. based on different testing methods) are independent from each other. Additionally, the specificity of the surveillance system is assumed to be 100% (Martin et al., 2007a). Thus, if a positive sample is found, further confirmatory testing is made in the herd to avoid false positive results (Martin et al., 2007a; Martin et al., 2007b).

With "fast" spreading pathogens, it can be assumed that P_U is quickly reached (Martin et al., 2007a). In contrast, BVDV can be considered, in some cases, as a "slowly" spreading virus, especially if it is introduced into large herds through TI animals (Moerman et al., 1993; Foddai et al., 2014a). Therefore, long time could pass before P_U is reached within a herd and before such a herd is detected by the surveillance system (long *HRP*).

For that reason, in this study, the temporal sensitivity of the surveillance system (Thurmond, 2003) was estimated, with *HRP* of 90 or 365 days. Those two time intervals, lead to the temporal *SSe* by testing quarterly and testing on yearly basis after BVDV introduction, respectively. Moreover, we investigated the effect of introducing one PI calf or one TI milking cow into naïve Danish dairy herd(s), on the final temporal *SSe*. The impact of using alternative testing strategies, such as changing test and/or using individual serum testing for herds at higher risk of BVDV introduction was also evaluated.

Herds were divided into two levels of risk: Herds with import of live cattle (*ImpoCattle* category) or without (*NoImpoCattle* category) (Fig. 1), based on a previous study (Foddai et al., 2014b).

We developed the stochastic scenario trees (Fig. 1, 2, and 3) in an Excel spreadsheet (Microsoft Office Excel 2007) using the software @Risk 6 (Palisade Corporation). The models were run for 10,000 iterations, with random seed and Latin Hypercube.

Finally, a sensitivity analysis was carried out to investigate, which inputs had the highest impact on the outputs.

2.2 Data analysis

We considered Danish dairy herds and beef herds as two distinct populations in the country (as in Foddai et al., 2014b). Thereafter, we focused on the Danish dairy population.

Data from 2010, on herd size, milk deliverance, imports/exports of live cattle, imports of semen and embryos, was obtained from the Danish Cattle Federation for all Danish dairy cattle herds. Information on hoof trimmers practicing in Denmark and abroad was based on a previous study (Foddai et al., 2014b). These data were used to estimate the *RR* of introducing BVDV for each risk category. Descriptive analyses were done using the freeware R (R Development Core Team, 2012).

2.3 Risk of infection per herd category

In the *NoImpoCattle* category, we included the following sources of infection in our model: import of semen and embryos, visits by contaminated trucks used abroad, and visits by hoof trimmers practicing in cattle herds in Denmark and in other countries.

In the *ImpoCattle* category, we included the same BVDV introduction routes plus imports of live cattle.

To quantitatively assess the annual risk of BVDV introduction in each risk category, we used a previously published stochastic model (Foddai et al., 2014b).

Foddai et al. (2014b) defined trucks used abroad for transports of live cattle, as a source of BVDV introduction into Danish dairy herds and estimated that the annual number of truck visits, which could lead to introduction of BVDV into Danish dairy herds is 5606. In each herd category, we assumed that the number of truck visits was proportional to the number of exports from each category (Table, 1).

Moreover, the annual number of hoof trimmer visits, which could led to BVDV introduction into each category, was estimated as shown in Table 1.

2.4 Probability of reaching the threshold prevalence (PTR) within the milking group

The threshold prevalence of antibody positive milking cows needed for BVD detection by BTM testing (or P_U within the milking group) was assumed to slowly increase over time. The probability of reaching this threshold prevalence (*PTR*) within a fixed *HRP* was estimated, by using a stochastic simulation model developed in R (Foddai et al., 2014a). This model was developed in order to simulate BVDV spread within a typical Danish dairy herd.

The *PTR* is affected by the route of BVDV introduction to the herd (PI or TI animal), the herd size, the threshold prevalence of the antibody ELISA used, and the time elapsed between BVDV introduction and day of testing (Foddai et al., 2014a). Thus, the *PTR* was calculated as the number of iterations out of 500, where the threshold prevalence was reached, at 90 or 365 days after the introduction of one PI calf or one TI milking cow, into a naïve Danish dairy herd.

In the *NoImpoCattle* category, herd sizes were between 1 and 1185 cows, while most of the herds were assumed to have around 150 cows. In the *ImpoCattle* category, the herd size was minimum 24, median 180 and maximum 1070 cows. Thus, the *PTR* of the minimum, median and maximum herd size within each category was estimated. This approach was repeated for each BVDV introduction route, test used and *HRP*. Then the PTR values from Table 2 were used in the stochastic scenario trees (Fig. 1, 2) in a Pert distribution ($PTR_{NoImpoCattle}$ and $PTR_{ImpoCattle}$) to represent the variability in the *PTR*, between herds of different sizes within each risk category.

2.5 Test parameters

We considered two ELISAs, which could be used to test BTM samples for antibodies against BVDV. These were the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997) and SVANOVIR®BVDV-Ab ELISA (Svanova Boehringer Ingelheim, Uppsala, Sweden) (Juntti et al.,1987; Niskanen et al., 1989; Niskanen et al., 1991; Niskanen, 1993). In a previous pilot study, we estimated the threshold prevalence for the Danish blocking ELISA to 50%. For the SVANOVIR ELISA, the threshold has been estimated to 6% (Niskanen, 1993).

Moreover, with a cut-off blocking % (bl%) of 50, the sensitivity (*Se*) of the Danish blocking ELISA on BTM is 100% (Houe, 1999), while for the SVANOVIR ELISA, the *Se* has been

estimated between 93.4% and 99.6% (Lindberg, 2000). In the latter case, a Uniform distribution ranging between the two extremes was used on the *Se.*

We assumed that the test *Se* could be achieved, when the threshold prevalence was reached. The same approach was used by Graat et al. (2001) for infectious bovine rhinotracheitis (IBR). When the threshold prevalence was not reached, we assumed that the BTM of an infected dairy herd was classified as false negative (Fig. 1).

2.6 Input parameters

We used data of last trimester of 2010, where approximately 4109 herds (*N*) delivered milk and were tested. The steps needed for a BVDV positive dairy herd, to give a positive BTM value are represented with nodes in the scenario tree in Fig. 1. Hence, as reference scenario, we considered one surveillance component based on BTM testing with the Danish blocking ELISA (Rønsholt et al., 1997; Bitsch et al., 1997). Such a component is comprehensive of the whole population of Danish dairy herds and represented the current surveillance system for the national dairy herd.

The first node of the scenario tree divided the population of tested dairy herds into the two risk categories: *NoImpoCattle* and *ImpoCattle* (Fig. 1). Thus, the proportion of dairy herds in the respective risk category was represented by $PrP_{NoImpoCattle}$ and $PrP_{ImpoCattle}$, respectively.

The probability that a herd was infected was represented by the effective probability of infection ($EPI_{NoImpoCattle}$ and $EPI_{ImpoCattle}$) (Fig. 1). The *EPIj* within each herd category "*j*" was obtained by multiplying the P_H with the adjusted RR_j (ARR_j). The RR_j estimates were adjusted to

maintain the specified relativity (weight) with the risk reference category and yet average to one over the whole population (Martin, 2008).

The *NoImpoCattle* category was used as risk reference category ($RR_{NoImpoCattle}$ = 1). Thus, the adjusted relative risk in that reference category was:

$$ARR_{NoImpoCattle} = \frac{1}{(PrPNoImpoCattle + RRImpoCattle * PrPImpoCattle)} \quad \text{(Eq. 1)}$$

The $RR_{ImpoCattle}$ was the risk of BVDV introduction in the *ImpoCattle* category, relative to the *NoImpoCattle* category (Section 3.2). The adjusted relative risk for the *ImpoCattle* category was:

$$ARR_{ImpoCattle} = ARR_{NoImpoCattle} \text{ x } RR_{ImpoCattle} \quad \text{(Eq. 2)}$$

The component unit sensitivity was then calculated as the weighted sum of the probabilities with a positive outcome at the end of each limb of the stochastic scenario tree (Fig. 1, Eq. 3). Thus, the overall probability (*SSe*) that at least one BTM positive herd was detected in the current BTM surveillance system was:

$$BTM_{SSe} = 1 - [1 - (PrP_{NoImpoCattle} \text{ x } EPI_{NoImpoCattle} \text{ x } PTR_{NoImpoCattle} \text{ x } Se + PrP_{ImpoCattle} \text{ x } EPI_{ImpoCattle} \text{ x } PTR_{ImpoCattle} \text{ x } Se)]^{N} \quad \text{(Eq. 3)}$$

2.7 Temporal SSe with different surveillance strategies.

The temporal *SSe*, was evaluated with different surveillance strategies. These included:

a) Current surveillance system (Fig. 1; Eq. 3) where all Danish dairy herds are tested with the Danish blocking ELISA.

b) Testing all BTMs in the indirect SVANOVIR ELISA instead of using the Danish blocking ELISA (Fig. 1; Eq. 3).

c) Using the blocking ELISA or d) the SVANOVIR, but testing BTM samples in *NoImpoCattle* herds (Fig. 2; Eq. 4) and individual serum in *ImpoCattle* herds (Fig. 3; Eq. 5).

Thus, in strategies "a" and "b" we proceeded as described in section 2.6, with the surveillance system represented by one single surveillance component (Fig. 1). In strategy "c" and "d", we considered two distinct surveillance components (one for each category).

Therefore, the temporal sensitivity in the BTM testing component (*BTMCSe*) was calculated as:

$$BTMCSe = 1- (1- EPI_{NoImpoCattle} \times PTR_{NoImpoCattle} \times Se) \wedge n1 \quad \text{(Eq. 4)}$$

Where *n1* was the number (4101) of dairy herds within the *NoImpoCattle* category (Table 1).

The temporal sensitivity for the individual serum testing component (*SerumCSe*) was calculated as:

$$SerumCSe = 1- (1- EPI_{ImpoCattle} \times PTR_{ImpoCattle} \times HSe) \wedge n2 \quad \text{(Eq.5)}$$

The *PTR* values used in Eq. 5 were estimated by setting the within herd design prevalence (P_U) to 10%. Thus, simulations made with the model by Foddai et al. (2014a) stopped, when 10% of cattle present in the herd (milking and not) seroconverted. The herd sensitivity (*HSe*) was assumed to be 95% for both tests and replaced the *Se* of the test used on BTM samples. Therefore, we assumed that according to the ELISA used, enough animals were sampled within

each *ImpoCattle* herd, to reach the mentioned *HSe*. Moreover, *n2* was the number of dairy herds (eight), which imported live cattle in 2010 (Table 1).

Thus, the overall temporal *SSe* of surveillance strategies "c" and "d" was estimated by combining the *CSes* from the two surveillance components as:

$$SSe = 1 - [(1 - BTMCSe) * (1 - SerumCSe)] \quad \text{(Eq. 6)}$$

2.8 Negative predictive value (NPV) of the surveillance system

The scenario tree methodology developed by Martin et al. (2007a; Martin et al., 2007b; Martin, 2008) is usually used to show complete freedom from a disease or from a pathogen (*PFree*) at country/area level. For that purpose, the negative predictive value (NPV or *PFree*) of the surveillance system is estimated to represent the confidence that a country, classified as free from a pathogen by the system, is truly free. In that case, the design prevalence (P_H and P_U) represents a hypothetical level of infection in the country. If a single positive unit is found, the country would lose the "free status" (Martin et al., 2007a; Martin et al., 2007b; Martin 2008).

In our study, we estimated the NPV of the surveillance system to show the *PFree*, but we also estimated the confidence (*PLow*) that the prevalence of infected herds is below the P_H. In the latter case, we did not exclude that a few positive herds could be present in the country.

Thus, to estimate *PLow* we used the temporal *SSe* based on P_H = 0.2% within Eq. 7, while to estimate *PFree* the temporal *SSe* was obtained using P_H = 0.02%.

The NPV of the surveillance system was estimated as (2007a; Martin et al., 2007b; Martin, 2008):

$$PLow\ (or\ PFree) = \frac{(1 - PriorPInf)}{(1 - PriorPInf) + (PriorPInf * (1 - SSe))} \quad (Eq. 7)$$

where *PLow* (or *PFree*) is the confidence that the prevalence of infected herds is below the assumed design prevalence 0.2% (or 0.02%), after the surveillance period. *PriorPInf* is the probability that the country is infected with the assumed design prevalence, at the beginning of the surveillance period. This input was set to 50%, which corresponds to a conservative uninformed prior (Martin et al., 2007a; Martin et al., 2007b).

Moreover, the *PriorPInf* was adjusted ($PriorPInf_{Adj}$) (Eq. 8), by taking into account the probability of BVDV introduction during the period of surveillance (*PIntro*).

$$PriorPInfAdj = PriorPInf + PIntro - (PriorPInf * PIntro) \quad (Eq. 8)$$

The annual median *PIntro* for dairy herds has been estimated as 10.7% (90% prediction interval: 1.7%; 36.6%) (Foddai et al., 2014b). When we estimated the *PLow* and *PFree* with *HRP* of 90 days, the annual *PIntro* was divided by 4. Hence, we assumed that the *PIntro* was similar between trimesters, though in reality some variations could be present (Foddai et al., 2014b).

PLow and *PFree* were estimated for each infection scenario (introducing a PI or a TI animal), surveillance strategy (a, b, c or d) and *HRP* (90 or 365 days).

2.9 Sensitivity analyses

The impact of the different inputs on the final estimates was studied, by using the tornado plot function in @Risk. The amount of change in the mean output due to a plus 1 standard deviation for each input was investigated. In this way a ranking of the inputs according to their importance could be made.

We used as reference scenario, all dairy herds tested with the Danish blocking ELISA (current system) one year after introduction of a PI calf into a single dairy herd (P_H = 0.02%), since PIs are usually considered to be the main source of BVDV spread (Niskanen et al., 2000) between and within cattle herds.

Moreover, for the same scenario, the temporal *SSe* and the related *PFree* were estimated setting the *Se* of the Danish blocking ELISA (Fig. 1) as a Uniform distribution between 89% and 100%. We estimated the lowest *Se* limit in a pilot study (unpublished data). Therefore, the impact of using a lower *Se* under the current surveillance strategy (a) was investigated.

3. Results

3.1. Data analysis

The imports of live animals, doses of semen, embryos, and the visits of trucks and hoof trimmers, are shown per herd category in Table 1. Cattle were only imported to eight herds in 2010, and therefore, all imported animals went to the *ImpoCattle* category. On the other hand, the annual quantity of imported semen and embryos; and the number of trucks and hoof trimmer visits was remarkably higher for the *NoImpoCattle* category than for the *ImpoCattle* category (Table, 1).

3.2. Risk assessment per herd category.

Results of the data analysis (Table, 1) were fed into the model developed by Foddai et al. (2014b) to estimate the risk of BVDV introduction from abroad per herd category. The median annual risk of BVDV introduction in the *NoImpoCattle* category was 4.8% (90% prediction interval 0.7%, 21.8%), while in the *ImpoCattle* category it was 5.1% (0.7%, 22.4%).

Based on those findings, the relative risk of BVDV introduction in the *ImpoCattle* category ($RR_{ImpoCattle}$) was calculated as the ratio between the two median risk estimates (and between their respective 90% prediction intervals). Thus, the $RR_{ImpoCattle}$ was simulated from a Pert distribution with minimum 1, mode 1.03 and maximum 1.07, to calculate the $ARR_{ImpoCattle}$ and the $EPI_{ImpoCattle}$ (Fig. 1, 2, and 3).

3.3. PTR values for testing strategies "a" and "b", according to infection scenario and HRP.

The *PTR* values were higher in small herds than in large herds, higher for BVDV introductions through one PI calf than through one TI milking cow, and higher with *HRP* of 365 days than with *HRP* of 90 days (Table 2). Moreover, the SVANOVIR ELISA had higher *PTR* values than the Danish blocking ELISA (Table 2).

In the *ImpoCattle* category, the *PTR* ranged from 0%, e.g. when a PI calf or a TI cow was introduced into a herd with 1070 cows, and the BTM was tested 90 days later with the blocking ELISA; to 97.2% when one PI calf was introduced into a herd of 24 cows, and the BTM was tested one year later with the SVANOVIR (Table, 2).

In the *NoImpoCattle* category the *PTR* ranged from 0%, e.g. when a PI calf or a TI cow was introduced into a herd of 1185 cows, and the BTM was tested 90 days later with the blocking ELISA; to 100% when one infectious animal was introduced into a herd of a single cow (Table, 2).

3.4. PTR values for surveillance strategy "c" and *d"*

The *PTR* ranged from 0%, when a PI calf or a TI cow was introduced into an *ImpoCattle* herd with 1070 cows, and individual serum was tested 90 days later; to 95.4% if a PI calf was introduced into a herd with 24 cows and the animals were tested 365 days after (Table 3).

3.5. Overall temporal SSe and related PLow (BVDV introduction in eight dairy herds)

With testing strategy "a" (Danish blocking ELISA), the median temporal *SSe* ranged from 64.4%, when a TI milking cow was introduced in at least eight dairy herds, and the BTM was tested in all Danish dairy herds 90 days later; to 98.0% if a PI calf was introduced, and the BTM was tested 365 days later (Table, 4). The related *PLow* estimates were 72.5% and 97.5%, respectively (Table 4).

For testing strategy "b" (SVANOVIR ELISA), the temporal SSe´s were similarly estimated to 63.5% and 99.8%, while the *PLow´s* were estimated to 71.9% and 99.7%, respectively (Table 4).

In the scenario where one TI cow was introduced and a *HRP* of 90 days was used, the *SSe* and the related *PLow* were slightly higher (around +1%) in surveillance strategy "a" than in strategy "b" (Table 4), because with both tests, detection would occur in the *NoImpoCattle* category if the

herd has one cow and so the *PTR* is 100%. With the SVANOVIR, the *PTR* was >0% (33%) also in the *ImpoCattle* herd with 24 cows (Table 2) and in the *NoImpoCattle* herd with 150 cows (0.20%). However, the *Se* of the SVANOVIR ELISA on BTM was assumed to be lower (Lindberg, 2000) than the *Se* of the Danish blocking ELISA (Houe, 1999).

In contrast, when a *HRP* of 365 days was used, surveillance strategy "b" gave higher *SSe* and *PLow* estimates than in strategy "a". In that case, *PTR* values were higher for both tests compared to the situation where a *HRP* of 90 was used, and were higher for the SVANOVIR than for the Danish blocking ELISA (Table, 2).

3.6. Overall temporal SSe and related PFree (BVDV introduction in a single dairy herd)

With strategy "a", the median temporal *SSe* ranged from 12.1%, e.g. when a TI milking cow was introduced into a dairy herd, and the BTM was tested in all Danish dairy herds 90 days later; to 38.7% if a PI calf was introduced, and the BTM was tested 365 days later (Table, 4). The related *PFree* estimates were 51.6% and 55.5%, respectively (Table 4).

For testing strategy "b", temporal SSe´s were similarly estimated to 11.9% and 53.7%, while the *PFree´*s were estimated to 51.5% and 62.4%, respectively (Table 4).

For the same reasons described in section 3.5, the *SSe* and the related *PFree* were slightly higher in surveillance strategy "a" than in strategy "b", if one TI cow was introduced and a *HRP* of 90 days was used (Table 4).

3.7. Overall temporal SSe and related PLow/PFree for testing strategies "c" and "d".

For strategy “c” and “d”, the *SSe* and the related *PLow/PFree* were less than 1% higher than in the other two surveillance strategies.

3.8. Sensitivity analysis and importance of input parameters.

According to the sensitivity analysis, the input with the highest impact on the estimated temporal *SSe* was the *PTR* distribution used in the *NoImpoCattle* category ($PTR_{NoImpoCattle}$). When this input was increased with 1 standard deviation the mean temporal *SSe* increased between 0 and 11.5%. The second input in order of importance was the *PTR* distribution used in the *ImpoCattle* category ($PTR_{ImpoCattle}$). In that case, the increase caused on the temporal *SSe* ranged between 0 and 0.03%. The other inputs caused a change lower than 0.03%.

For the *PFree*, the most important input was still the $PTR_{NoImpoCattle}$. Increasing such an input with one standard deviation caused an increase in the mean *PFree* between 0 and 4.5%. The second most important input was the annual (overall) probability of BVDV introduction (*PIntro*) in Danish dairy herds. Increasing the *PIntro* of 1 standard deviation caused a decrease in the *PFree* between 0 and 3.1%. All the other inputs caused a change lower than 0.006%.

When we decreased the *Se* of the Danish blocking ELISA on BTM, the $PTR_{NoImpoCattle}$ was still the most important parameter for the temporal *SSe*. The *Se* of the blocking ELISA was the second most important input. Increasing the latter of one standard deviation caused an increase in the temporal *SSe* between 0 and 1%. The overall temporal *SSe* was approximately 2% lower than the estimates reported in Table 4 (see estimates under “blocking_365” and “1 PI introduced”). For the *PFree*, the *Se* of the Danish blocking ELISA was the third most important input, after the

$PTR_{NoImpoCattle}$ and *PIntro*. Increasing of one standard deviation the *Se* caused an increase between 0 and 0.4% in the *PFree*. All other inputs caused a change lower than 0.4%.

4. Discussion

4.1. A new approach – Including the PTR in the stochastic scenario tree methodology

In previous studies, where the stochastic scenario tree methodology was used, the "diagnostic sensitivity" of the surveillance system was estimated (Martin et al., 2007a, Martin et al., 2007b; Blickenstorfer et al., 2011). Moreover, the methodology developed by Martin et al. (2007a; Martin et al., 2007b; Martin, 2008) is usually used to show complete freedom from a pathogen/disease. However, as stated by Sergeant et al. (2010) "disease freedom does not necessarily imply the total and complete absence of a disease causing agent".

We followed the concepts from Martin et al. (2007a; Martin et al., 2007b; Martin, 2008). Additionally, we estimated the temporal *SSe* and the related negative predictive value (Eq. 7) of the surveillance system, to substantiate the confidence (*PFree*) in complete BVDV freedom (< 1 infected herd), and the confidence (*PLow*) in low herd prevalence (< 8 infected herds).

The way we adapted the scenario tree models allowed us to estimate the "temporal sensitivity" of the surveillance system. Thurmond (2003) suggested that the temporal sensitivity is affected by the disease transition state sensitivity of the test used. This means that the sensitivity of the test is affected by the time elapsed since a herd (or an animal) became infected. In our case, the transition state herd sensitivity of the ELISA used, was conditioned upon the immune status of the milking flock on the day of sampling, and of the entire *ImpoCattle* herd for surveillance

strategies “c” and “d”. That status varied in time according to *HRP*, BVDV introduction route (with PI or TI animals) and herd size. Uncertainty, due to these variables was included in our estimates (Tables 4), by using the *PTR* parameter in the scenario trees, between the infection node “Herd infection status” and the detection node “ELISA” (Fig. 1, 2 and 3).

In this way, we could evaluate if the surveillance system can actually function as an early-warning system, or if optimization was needed, to increase the probability of detecting infected herds (*SSe*) within the aimed time period. To our knowledge, this is the first study, where the impact of the *HRP* is included in the evaluation of a surveillance system using stochastic scenario trees.

We believe that these observations should be considered, especially when early-warning surveillance systems are established for slowly spreading diseases, as is the case of BVD in large dairy herds after introduction of TI animals (Moerman et al., 1993; Moen et al., 2005; Foddai et al., 2014a).

4.2 Temporal SSe and related PLow

Conclusions on surveillance sensitivity and disease status at national level should be related to a specific time period, when the pathogen could have been introduced into the country. Hence, in our case, the temporal *SSe* and *PLow/PFree* should be related to the period when the BVDV could have been introduced into the Danish dairy herd(s).

Between December 2011 and December 2012, no dairy herds have been found positive in Denmark. Therefore we can assume that, very few herds (≤ 8) or no herds were infected in the country at the beginning of 2012.

If BVDV was introduced by a PI calf in at least 8 dairy herds, the median probability of detecting at least one of these herds after a year period, by BTM testing, would be > 95% with both ELISAs (which could be considered as an acceptable level of confidence). The confidence in low herd prevalence (*PLow*) would be high as well (Table, 4). This means that, if the aim of the surveillance system was to substantiate on annual basis that the prevalence of herds infected by at least one PI animal is <0.2%, there is no need to replace the Danish blocking ELISA with the SVANOVIR ELISA.

On the other hand, if the objective of the surveillance system was to detect BVD 90 days after introduction of a PI calf in 0.2% dairy herds, then the SVANOVIR ELISA would be preferred, because only that test showed median temporal *SSe* and *PLow* higher than 95% (Table, 4). PI animals are usually considered to be the major sources of BVDV spread (Niskanen et al., 2000), between and within cattle herds, and considering only BVDV introductions by those animals could therefore be sufficient.

In contrast, if the objective of the surveillance system was to detect BVD after introduction of a TI cow in 0.2% dairy herds, the SVANOVIR ELISA could be used, but in that case, the median temporal *SSe* and the related *PLow* would be below 95% despite the chosen test and *HRP* (Table, 4).

4.3 Temporal SSe and related PFree

According to the BTM testing made in the fourth trimester of 2010 (with the Danish blocking ELISA), it can be concluded that the probability of detection one year after one single herd was infected by a PI calf (P_H = 0.02%), was low (Table, 4). Therefore, at the end of 2010, the confidence in complete freedom (*PFree*) was also low.

If the SVANOVIR ELISA had been used (strategy "b"), under the same infection/*HRP* scenarios, the *SSe* and the related *PFree* would have been higher than with the Danish blocking ELISA (Table 4).

Moreover, we showed that, if one TI cow was introduced to a herd and a *HRP* of 90 days was used, the *SSe* and the related *PFree* were slightly higher in surveillance strategy "a" than in strategy "b" (Table 4). When a *HRP* of 365 days was used the contrary was observed. Those findings suggest that, when the herd size is very small (e.g. <50 cows) and the *HRP* is short, the temporal *SSe* becomes more dependent from the *Se* of the test used on BTM, than from the *PTR*. In fact, in small herds, even a high threshold prevalence of 50% can be reached in a short *HRP* (high *PTR*) (Table, 3 and 4). This was the case in Denmark in 1994, when the eradication program was launched. At that period, the average herd size in Danish dairy herds was 42 cows (Bitsch and Rønsholt, 1995), while currently it has increased to approximately 150 cows.

Since the size of Danish dairy herds is continuously increasing, the *PTR* value has higher importance in the current situation than in the past. With larger herds, tests that can detect a lower prevalence of seropositive animals, in a short *HRP* and with higher *PTR* should be preferred. This is the case of the SVANOVIR compared to the blocking ELISA. Using the former, a higher temporal *SSe* would be achieved, compared to the current surveillance system.

Furthermore, it must be noted that once that the threshold prevalence has been reached, increasing the BTM testing frequency would increase the costs due to the higher number of samples tested, but also the probability of detection (*SSe*). In contrast, using a higher BTM testing frequency, before the threshold prevalence is reached in the milking group, would be inefficient.

4.4 Surveillance strategies "c" and "d".

For surveillance strategies "c" and "d", although the *PTR* values were higher for individual serum testing than for BTM testing (with the blocking ELISA) in *ImpoCattle* herds (Table 2 and 3), the overall *SSe* and related *PFree/PLow* were not affected remarkably (Table 4), since the *RR* in the *ImpoCattle* category was close to 1. Hence, targeting herds at higher risk of infection by individual serum testing (with higher sampling costs) would not improve the surveillance system markedly.

4.5 Impact of HRP and kind of infectious animal introduced to the herd (PI vs. TI) on SSe and related PLow/PFree

We showed that, the *SSe* and the *PLow/PFree* were higher for *HRP* of 365 days than for *HRP* of 90. This was due to two main reasons: 1) the longer the time an infectious animal is kept in

the herd, the higher the probability that such an animal causes an outbreak [(4)], and 2) within the first 90 days from BVDV introduction, no new PI calves can be born in the herd.

The first observation is also valid for other pathogens, while the second is peculiar of the epidemiology of BVD. In fact, PI calves are born from susceptible cows which become infected within the first four months of pregnancy (120 days) (McClurkin et al., 1984; Brownlie et al., 1987; Baker, 1990). Hence, secondary PI calves will be born in the herd at least 5 months after introduction of the first infectious animal (PI or TI in our infection scenarios), because the cattle pregnancy lasts around 280 days.

Moreover, the first introduced infectious animal could be removed accidentally by the farmer (e.g. a male calf born from an imported pregnant cow). In that case, BVD could remain within the herd in a latent phase, due to PI calves carried by newly infected Trojan cows. Those cows do not shed virus after they seroconvert, and virus spread will start again in the herd after birth of the PI calves (Lindberg and Alenius, 1999). When PIs are present, the immunization of animals occurs quicker than when only TI animals are present (Foddai et al., 2014a), and detection of BVD infected herds by BTM testing becomes more likely. Especially in small herds, with the presence of PI animals, the risk of seroconversion during a six months period could be as high as 97% (Houe and Meyling, 1991).

When only TI animals are introduced, it is less likely that an outbreak occurs within the herd (Niskanen et al., 2000). Furthermore, even if an outbreak is caused by TIs, the time needed for detection is longer than with PIs, because the force of infection of the former is lower. In fact, in

[4] Here the term "outbreak" is used for herd infections where sufficient spread of BVDV occurs within the herd (enough infected animals), so that the threshold prevalence is reached within the milking group.

simulations studies, it is usually assumed that the within group transmission rate of TIs is approximately 16 times lower than in PIs, and that only the latter are able to spread BVDV between animals groups (e.g. from calves to milking cows) (Viet et al., 2004; Ezanno et al., 2007; Foddai et al., 2014a). Hence, the number of newly infected animals per unit of time is higher with PI cattle.

For these reasons, in most of the iterations where a TI was introduced and the within herd BVDV spread was simulated, the outbreak died out (self-clearance) (Lindberg and Alenius, 1999), before the threshold prevalence was reached. Thus, in those scenarios, the temporal *SSe* was low due to the low *PTR* values (Table 2 and 4). Further studies should be carried out to investigate the risk of spreading BVDV to other herds (e.g. by animal movements), before the first infected herd(s) is detected and/or before self-clearance occurs.

4.6 Sensitivity analysis

In the sensitivity analysis, we confirmed that the *PTR* is an important parameter to consider, when the temporal *SSe* of the surveillance system is estimated.

Moreover, we showed that the probability of BVDV introduction in the country (*PIntro*) could cause a decrease in the *PFree*. Testing imported animals at the border, could reduce the *PIntro* (Foddai et al., 2014b) and could increase the confidence in freedom (*PFree*), despite of the low temporal *SSe*. Then, the latter would become less important, if risk mitigation measures were improved at the border.

For the Danish blocking ELISA, setting *Se* lower that 100%, did not change the temporal *SSe* and the *PFree* remarkably. Thus, the choice of this input had not an important impact on our conclusions.

4.7 Limitations of the study

Our estimates (*SSe*, *PFree* and *PLow*) could be considered as conservative, since we assumed that herds become infected by introduction of one BVDV positive animal only (a PI or a TI). In reality, more infected animals could be introduced to one herd at the same time. When more infectious animals are introduced into the herd, the *PTR* values (and so the temporal *SSe*) will be higher than we estimated, because more virus would be shed within the herd, more milking cows become immune in a shorter time period and detection can occur with higher chances and sooner.

Moreover, the *PTR* values were estimated for three herd sizes within each risk category. A more precise modeling for all herd sizes would have required to run the simulation at least 1185 times, to determine the *PTR* for each herd size in the country. Because this was not feasible, we set *PTR* values as Pert distributions within each risk category. Therefore, the herd size within each category did not match perfectly with the respective *PTR*.

Finally, we assumed that detection by BTM testing could occur when a fixed threshold prevalence of antibody positive milking cows was reached within a herd, and that, these cows had similar milk production and antibody levels in milk. The same approach was used by Graat et al. (2001) for IBR. In reality, some seroconverted animals might have high antibody titers in milk, e.g. because they carried PI calves (Lindberg et al., 2001). In that case, infected herds

could be detected earlier with higher probability, than we estimated. However, higher serum antibody levels in PI carrier cows (or Trojan cows) have been shown mainly in the last two months of pregnancy (Lindberg et al., 2001), when cows are usually dry and do not contribute to the BTM. Moreover, according to Brownlie et al. (1998), the level of antibodies in serum of Trojan cows, rapidly decreases after calving. Further studies are needed to investigate how the sensitivity of the ELISA used on BTM samples changes per day (after the introduction of infectious animals), according to prevalence of seroconverted milking cows, their individual milk production and antibody titer.

5. Conclusion

Using the SVANOVIR ELISA on BTM, would increase the temporal sensitivity and the related confidence in BVD freedom (and in low herd prevalence), compared to the current situation, where the Danish blocking ELISA is used. Individual serum testing in the few dairy herds importing cattle would not remarkably increase the temporal *SSe* and the related *PLow/PFree*.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Food, Agriculture and Fisheries of Denmark (Grant number 3412-09-02603) and by the Danish Cattle Federation. We would like to thank Dr. Peter Lind for his critical reading of the manuscript.

References

Baker, J.C., 1990. Clinical aspects of bovine virus diarrhoea virus infection. Rev. Sci. Tech. Off. Int. Epiz. 9, 25-41.

Bitsch, V., and Rønsholt, L., 1995. Control of bovine viral diarrhea virus infection without vaccines. Vet. Clin. N. Am-Food A. 11, 627-640.

Bitsch, V., Houe, H., Nylin, B., Rønsholt, L., 1997. Examination of blood and bulk tank milk samples to monitor the bovine virus diarrhoea infection status of cattle herds. In: 3rd ESVV Symposium on Pestivirus Infections, Lelystad, The Netherlands, 158–161.

Blickenstorfer, S., Schwermer, H., Engels, M., Reist, M., Doherr, M.G., and Hadorn D.C., 2011. Using scenario tree modelling for targeted herd sampling to substantiate freedom from disease. Vet. Res. doi:10.1186/1746-6148-7-49

Brownlie J., Clarke M.C., Howard C.J., and Pocock D.H., 1987. Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann. Rech. Vet. 18, 157-166.

Brownlie, J., Hooper, L.B., Thompson, I., Collins, M.E., 1998. Maternal recognition of foetal infection with bovine virus diarrhoea virus (BVDV) - the bovine pestivirus. Clin. Diagn. Virol. 10, 141-150.

Collett, M.S., Anderson, D.K., and Retzel, E., 1988. Comparisons of the pestivirus bovine viral diarrhoea virus with members of the Flaviviridae. J. Gen. Virol. 69, 2637-2643.

Ezanno, P., Fourichon, C., Viet, A.-F., Seegers, H., 2007. Sensitivity analysis to identify key-parameters in modelling the spread of bovine viral diarrhoea virus in a dairy herd. Prev. Vet. Med. 80, 49-64.

Foddai, A., Enøe, C., Krogh, K., Stockmarr, A., Halasa, T., 2014a. Stochastic simulation modeling to determine time to detect bovine viral diarrhea antibodies in bulk tank milk. Prev. Vet. Med. in press. doi:10.1016/j.prevetmed.2014.07.007

Foddai, A., Boklund, A., Stockmarr, A., Krogh, K., Enøe, C., 2014b. Quantitative assessment of the risk of introduction of bovine viral diarrhea virus in Danish dairy herds. Prev. Vet. Med. 116, 75-88.

Graat, E.A.M., de Jong, M.C.M., Frankena, K., Franken, P., 2001. Modelling the effect of surveillance programmes on spread of bovine herpesvirus 1 between certified cattle herds. Vet. Microbiol. 79, 193-208.

Haigh, J.C., Mackintosh, C., and Griffin, F., 2002. Viral, parasitic and prion diseases of farmed deer and bison. Rev. Sci. Tech. Off. Int. Epiz. 21, 219-248.

Hoinville, L.J., Alban, L., Drewe, J.A., Gibbens, J.C., Gustafson, L., Häsler, B., Saegerman, C., Salman, M., Stärk, K.D.C., 2013. Proposed terms and concepts for describing and evaluating animal-health surveillance systems. Prev. Vet. Med. 112 (2013), 1-12.

Horst, H.S., Huirne, R.B.M., and Dijkhuizen, A.A., 1997. Risks and economic consequences of introducing classical swine fever into The Netherlands by feeding swill to swine. Rev. Sci. Tech. Off. Int. Epiz. 16, 207-214.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet. Microbiol. 64, 89–107.

Houe, H., and Meyling, A., 1991. Prevalence of bovine virus diarrhoea (BVD) in 19 Danish dairy herds and estimation of incidence of infection in early pregnancy. Prev. Vet. Med. 11, 9-16.

Juntti, N., Larsson, B., and Fossum, C., 1987. The use of monoclonal antibodies in enzyme linked immunosorbent assays for detection of antibodies to bovine viral diarrhoea virus. J. Vet. Med. B 34, 356-363.

Lindberg, A., 2000. Validation of an antibody ELISA used on bulk milk to screen dairy herds for bovine viral diarrhoea virus infection. In: Proceedings of the 9th International Symposium on Veterinary Epidemiology and Economics. Available at www.sciquest.or.nz

Lindberg, A.L.E, Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet. Microbiol. 64, 197-222.

Lindberg, A., Groenendaal, H., Alenius, S., Emanuelson, U., 2001. Validation of a test for dams carrying foetuses persistently infected with bovine viral-diarrhoea virus based on determination of antibody levels in late pregnancy. Prev. Vet. Med. 51, 199-214.

Martin, P.A.J., 2008. Current value of historical and ongoing surveillance for disease freedom: Surveillance for bovine Johne's disease in Western Australia. Prev. Vet. Med. 84, 291–309.

Martin, P.A.J., Cameron, A.R., Greiner, M., 2007a. Demonstrating freedom from disease using multiple complex data sources 1: a new methodology based on scenario trees. Prev. Vet. Med. 79, 71-97.

Martin, P.A.J., Cameron, A.R., Barfod, K., Sergeant, E.S.G., Greiner, M., 2007b. Demonstrating freedom from disease using multiple complex data sources 2: Case study- Classical swine fever in Denmark. Prev. Vet. Med. 79, 98-115.

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., and Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Can. J. Comp. Med. 48, 156-161.

Moen, A., Sol, J., Sampimon, O., 2005. Indication of transmission of BVDV in the absence of persistently infected (PI) animals. Prev. Vet. Med. 72, 93-98.

Moerman, A., Straver, P.J., de Jong, M.C.M., Quak, J., Baanvinger, T., van Oirschot, J.T., 1993. A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. Vet. Rec. 132, 622-626.

Niskanen, R., 1993. Relationship between the levels of antibodies to bovine viral diarrhoea virus in bulk tank milk and the prevalence of cows exposed to the virus. Vet. Rec. 133, 341-344.

Niskanen, R., Alenius, S., Larsson, B., and Juntti, N., 1989. Evaluation of an enzyme-linked immunosorbent assay for detection of antibodies to bovine virus diarrhoea virus in milk. J. Vet. Med. B 36, 113-118.

Niskanen, R., Alenius, S., Larsson, B., and Jacobsson, S.-O., 1991. Determination of level of antibodies to bovine virus diarrhoea virus (BVDV) in bulk tank milk as a tool in the diagnosis and prophylaxis of BVDV infections in dairy herds. Arch. Virol. (Suppl. 3), 245–251.

Niskanen, R., Lindberg, A., Larsson, B., and Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Vet. Scand. 41, 93-99**.**

OIE, 2010. Enzootic bovine leucosis. Chapter 11.9. Article 11.9.2. Terrestrial Animal Health Code.

OIE, 2010. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis. Chapter 11.11. Article 11.11.2. Terrestrial Animal Health Code.

OIE, 2013. Bovine tuberculosis, Chapter 11.6. Article 11.6.2. Terrestrial animal health code.

Olafson, P., MacCallum, A.D., and Fox, F.H., 1946. An apparently new transmissible disease of cattle. Cornell Vet. 36, 205-213.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extintion. Vet. Res. 41:44, 1-14. DOI: 10.1051/vetres/2010016

Rønsholt, L., Nylin, B., Bitsch, V., 1997. A BVDV antigen- and antibody blocking ELISA (DVIV) system used in a Danish voluntary eradication program. In: 3rd ESVV Symposium on Pestivirus Infections, Lelystad, The Netherlands, 150–153.

Sergeant, E., Happold, J., Hutchison, J., Langstaff, I., 2010. Evaluation of Australian surveillance for freedom from bovine tuberculosis. Report prepared for the Australian biosecurity CRC for emerging infectious disease; AusVet Animal Health Services 1-42.

Thurmond, M.C, 2003. Special article: Conceptual foundations for infectious disease surveillance. J. Vet. Diagn. Invest. 15, 501-514.

Uttenthal, Å., Stadejek, T., and Nylin, B., 2005. Genetic diversity of bovine viral diarrhoea viruses (BVDV) in Denmark during a 10-year eradication period. APMIS, 113, 536-541.

Viet, A.-F., Fourichon, C., Seegers, H., Jacob, C., Guihenneuc-Jouyaux, C., 2004. A model of the spread of the bovine viral-diarrhoea virus within a dairy herd. Prev. Vet. Med. 63, 211-236.

Table 1. Results of data analysis and risk assessment for herds with import of live cattle (*ImpoCattle*) and without (*NoImpoCattle*). N = number of dairy herds delivering milk and tested in the fourth trimester of 2010 within each category, PrP = proportion of herds within each risk category, EPI = effective probability of infection in each risk category.

Herd Category	**N**	**Imported live cattle**	**Semen doses**	**Embryos**	**Truck visits**	**Hoof trimmer visits**	**PrP**	**EPI[d]**
ImpoCattle	8	246	3,776	5	5606 * 0.8% [b] = 45	[c] A*B* 0.20%	0.20%	0.025%
NoImpoCattle	4101	0	301,020	272	5606 * 99.2% [b] = 5561	[c] A*B* 99.80%	99.80%	0.024%
Total	4109	246[a]	304,796[a]	277[a]	5606[a]		100%	

[a,] From Foddai et al. (2014b)

[b,] According to Foddai et al. (2014b), in total 5606 truck visits at risk occur in Danish dairy herds during a one-year period. The percentage of exports from the the *ImpoCattle* and the *NoImpoCattle* category was 0.8% and 99.2%, respectively (Danish data 2010). We assumed that the number of trucks visits at risk in each category was proportional to the exports occurred from the category.

[c] The number of hoof trimmers visiting cattle herds abroad (A) during a one-year period was Pert (5, 7, 18), while the number of times each hoof trimmer crosses the border (B) was Pert (1, 8, 30) (from Table 8 in Foddai et al., 2014b). The annual number of hoof trimmer visits, which could lead to BVDV introduction into each category, was estimated by: A * B * $PrP_{ImpoCattle}$ and A * B * $PrP_{NoImpoCattle}$.

[d] The EPI reported in the table was calculated using P_H 0.02%. When we used P_H 0.2% the *EPI* was 0.20 for the *ImpoCattle* category and 0.19% for the *NoImpoCattle*.

Table 2. Number of iterations out of 500 (%), where the threshold prevalence of positive milking cows was reached (*PTR*) in herds of different size, using the stochastic within herd simulation model by Foddai et al. (2014a) and according to sampling day (*HRP* of 90 or 365 days), BVDV introduction route (PI calf or TI cow), test used (blocking ELISA or SVANOVIR) and herd size within each category (*ImpoCattle* or *NoImpoCattle*).

			PTR with 1 PI		**PTR with 1 TI cow**	
Herd category	**Test**	**Herd size (in cows)**	**HRP = 90 days**	**HRP = 365 days**	**HRP = 90 days**	**HRP = 365 days**
ImpoCattle	blocking ELISA	24	175 (35.0)	343 (68.6)	0 (0.0)	23 (4.6)
		180	0 (0.0)	206 (41.2)	0 (0.0)	2 (0.4)
		1070	0 (0.0)	14 (2.8)	0 (0.0)	0 (0.0)
	SVANOVIR	24	468 (93.6)	486 (97.2)	165 (33.0)	308 (61.6)
		180	240 (48.0)	376 (75.2)	0 (0.0)	38 (7.6)
		1070	3(0.6)	281 (56.2)	0 (0.0)	9 (1.8)
NoImpoCattle	blocking ELISA	1	500 (100.0)	500 (100.0)	500 (100.0)	500 (100.0)
		150	0 (0.0)	242 (48.4)	0 (0.0)	3 (0.60)
		1185	0 (0.0)	8 (1.60)	0 (0.0)	0 (0.0)
	SVANOVIR	1	500 (100.0)	500 (100.0)	500 (100.0)	500 (100.0)
		150	288 (57.6)	400 (80.0)	1 (0.20)	28 (5.60)
		1185	1(0.20)	278 (55.6)	0 (0.0)	12 (2.40)

Table 3. *PTR* values estimated for surveillance strategy "c" and "d", where individual serum is tested in *ImpoCattle* herds of different size. In that case, the *PTR* represents the number of iterations out of 500 (%), where 10% (P_U) prevalence of seroconverted animals (milking and not) was reached within the herd, according to sampling day (*HRP* of 90 or 365 days) from BVDV introduction in the herd (PI calf or TI cow).

	***PTR* with 1 PI**		***PTR* with 1 TI cow**	
Herd size (in cows)	***HRP* = 90 days**	***HRP* = 365 days**	***HRP* = 90 days**	***HRP* = 365 days**
24	473 (94.6)	477 (95.4)	6 (1.2)	44 (8.8)
180	215 (43.0%)	360 (72.0)	0 (0.0)	30 (6.0)
1070	0 (0.0)	265 (53.0)	0 (0.0)	4 (0.8)

Table 4. Median temporal *SSe* as % (90% prediction interval) with related confidence in low herd prevalence (*PLow*), and confidence in complete freedom from BVD (*PFree*), by testing all dairy herds in the BTM (Fig. 1) with the Danish blocking ELISA (strategy "a") or with the SVANOVIR ELISA (strategy "b") at 90 (blocking_90; SVANOVIR_90) or 365 days (blocking_365; SVANOVIR_365) after introduction of one PI calf or one TI milking cow. The design herd prevalence (P_H) was set to 0.2% (8/4109 infected herds) or 0.02% (1/4109 infected herds).

		1 PI introduced		**1 TI introduced**	
	ELISA_HRP	***SSe***	***PLow***	***SSe***	***PLow***
P_H = 0.2%	blocking_90	64.5 (7.9; 97.3)	72.4 (50.4; 97.2)	64.4 (7.8; 97.3)	72.5 (50.4; 97.2)
	SVANOVIR_90	98.6 (82.4; 99.9)	98.6 (84.9; 99.8)	63.5 (7.8; 97.0)	71.9 (50.4; 96.8)
	blocking_365	98.0 (78.1; 99.8)	97.5 (77.7; 99.8)	65.6 (8.5; 97.4)	69.0 (45.0; 96.7)
	SVANOVIR_365	99.8 (99.4; 99.9)	99.7 (99.2; 99.9)	73.8 (26.2; 97.8)	74.5 (50.5; 97.2)
		1 PI introduced		**1 TI introduced**	
	ELISA_HRP	***SSe***	***PFree***	***SSe***	***PFree***
P_H = 0.02%	blocking_90	12.1 (1.0; 36.2)	51.6 (48.1;59.5)	12.1 (1.0; 36.2)	51.6 (48.2;59.6)
	SVANOVIR_90	41.5 (20.1; 56.0)	61.5 (53.8; 68.0)	11.9 (1.0; 35.4)	51.5 (48.2; 59.1)
	blocking_365	38.7 (17.3; 55.1)	55.5 (46.1; 64.3)	12.5 (1.1; 36.6)	47.2 (44.1; 55.8)
	SVANOVIR_365	53.7 (46.8; 59.4)	62.4 (55.8; 67.5)	15.4 (3.7; 37.9)	48.0 (40.7; 56.3)

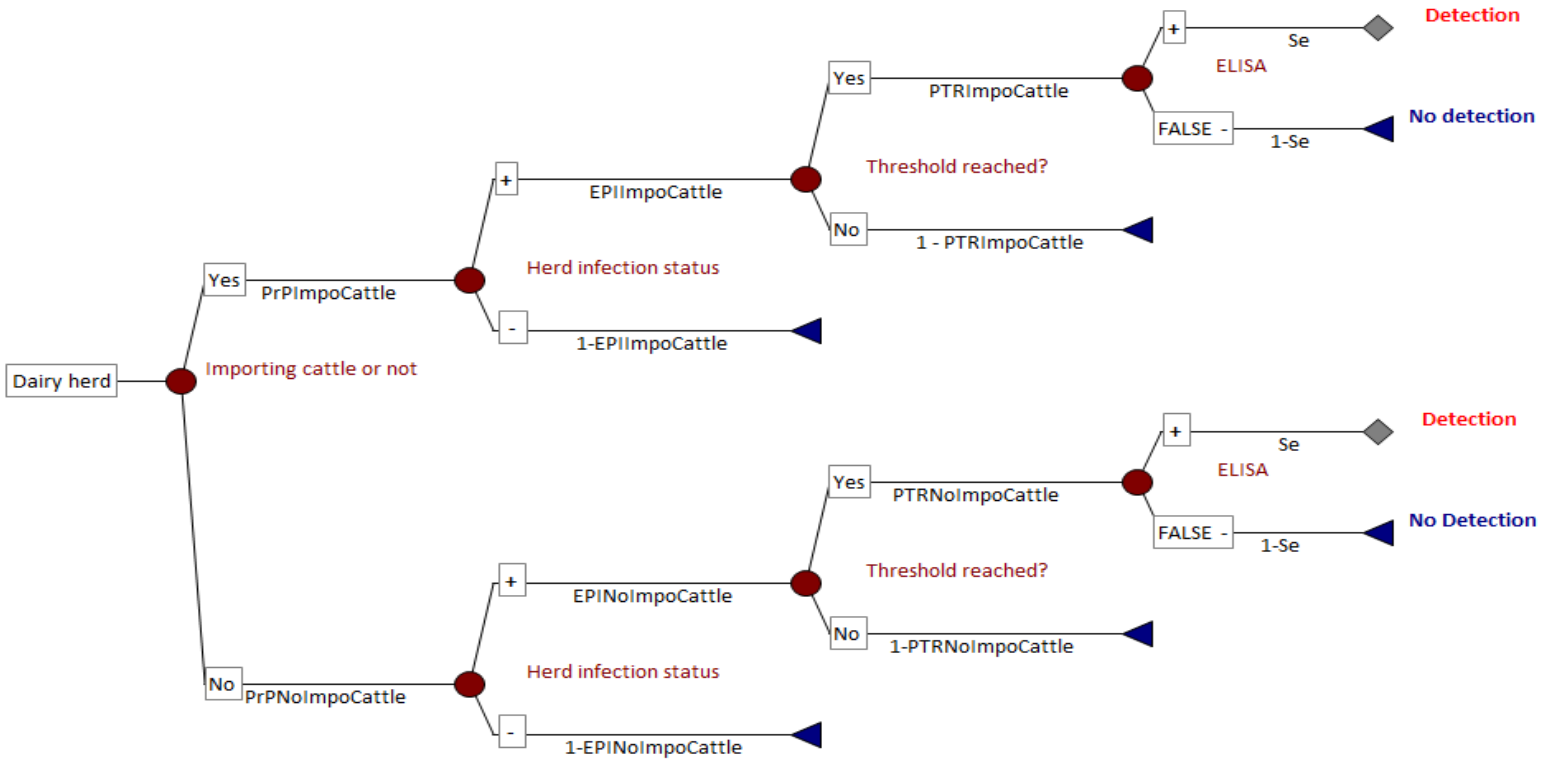


Figure 1. Stochastic scenario tree for the comprehensive surveillance component where all Danish dairy herds are tested in BTM (strategy "a" and "b"). $PrP_{ImpoCattle}$ and $PrP_{NoImpoCattle}$ = proportion of dairy herds within the *ImpoCattle* and *NoImpoCattle* category. $EPI_{ImpoCattle}$ and $EPI_{NoImpoCattle}$ = effective probability of infection within the *ImpoCattle* and *NoImpoCattle* category. $PTR_{ImpoCattle}$ and $PTR_{NoImpoCattle}$ = probability that the threshold prevalence is reached within the milking paddock at 90 or 365 days from BVDV introduction within herds of the *ImpoCattle* and *NoImpoCattle* category (Pert distributions based on Table 2). *Se* = Sensitivity of the antibody ELISA used (Danish blocking ELISA or SVANOVIR) on BTM, when the threshold prevalence of seroconverted milking cows is reached.

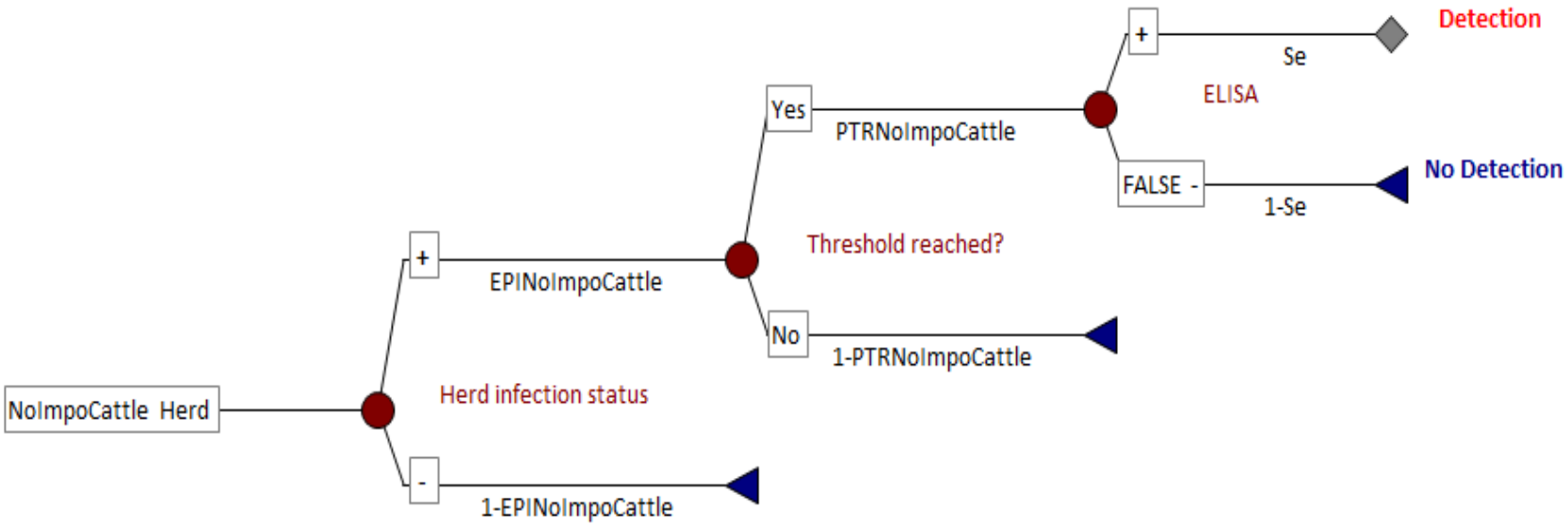


Figure 2. Stochastic scenario tree for the surveillance component of *NoImpoCattle* herds tested on BTM (surveillance strategy "c" and "d"). Legend as in Fig. 1. In that case, the node "Importing cattle or not" is not needed, since in this tree we only consider herds which did not import live animals.

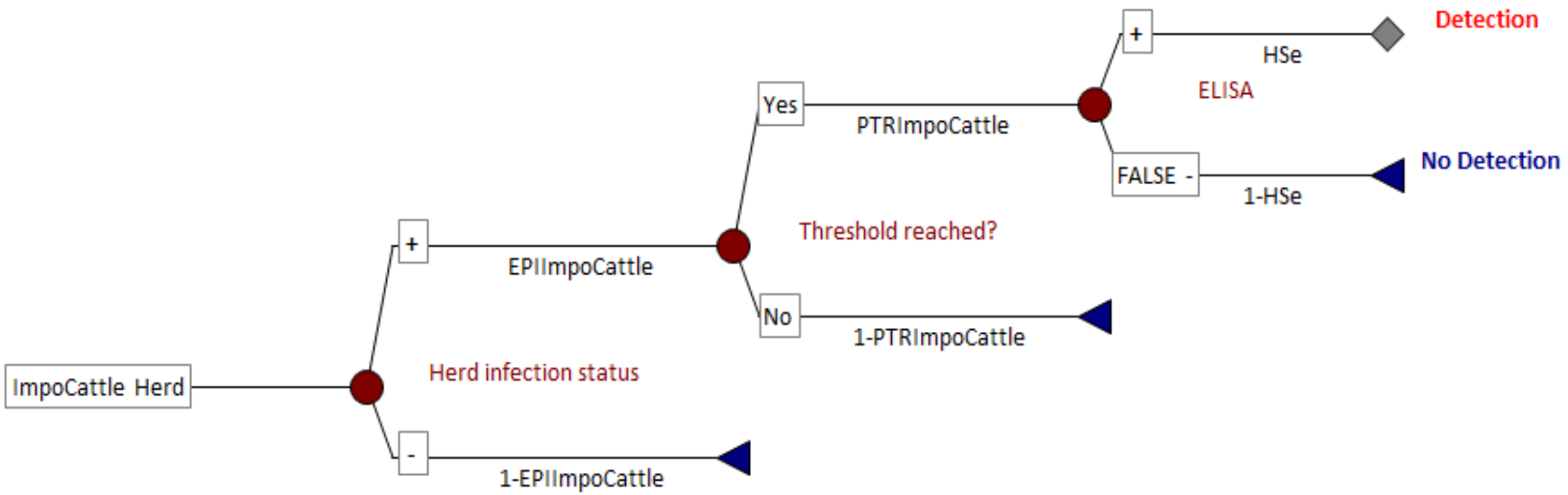


Figure 3. Stochastic scenario tree for the surveillance component of *ImpoCattle* herds tested on individual serum samples (in surveillance strategy "c" and "d"). $EPI_{ImpoCattle}$ = effective probability of infection in the category. $PTR_{ImpoCattle}$ = probability that the threshold prevalence (10%) is reached within the overall herd, at 90 or 365 days from BVDV introduction (Pert distribution based on Table 3). *HSe* = herd sensitivity to find at least one seroconverted animal at the within herd prevalence of 10%. The *HSe* was assumed to be the same for the Danish blocking ELISA and the SVANOVIR ELISA.

Appendix, list of abbreviations:

Abbreviation	Meaning
ARRj	Adjusted relative risk of infection in the "*j*" risk category.
BTM	Bulk tank milk
BTMCSe	Temporal sensitivity for surveillance component based on BTM testing
BTMSSe	Temporal surveillance system sensitivity when all dairy herds in the country are tested in bulk milk
BVD	Bovine viral diarrhea disease
BVDV	Bovine viral diarrhea virus
$EPI_{ImpoCattle}$	Effective probability of infection within the *ImpoCattle* category
$EPI_{NoImpoCattle}$	Effective probability of infection within the *NoImpoCattle* category
HRP	High risk period
IBR	Infectious bovine rhinotracheitis
ImpoCattle	Dairy herds which import live cattle
NoImpoCattle	Dairy herds which do not import live cattle
NPV	Negative predictive value of the surveillance system
PFree	Confidence in complete freedom from BVD (PH < 0.02% or <1/4109 infected herds)
P_H	Between herds design prevalence

PI	Persistently infected cattle
PIntro	Overall annual probability of BVDV introduction in the country
PLow	Confidence in low herd prevalence (PH < 0.2% or < 8/4109 herds)
PriorPInf	Prior probability that the country is infected at the assumed between-herds and within-herd design prevalence at the beginning of the surveillance period
$PrP_{ImpoCattle}$	Proportion of dairy herds which import live cattle
$PrP_{NoImpoCattle}$	Proportion of dairy herds which do not import live cattle
$PTR_{ImpoCattle}$	Probability that the threshold/design prevalence is reached within the *ImpoCattle* herds on the day of testing
$PTR_{NoImpoCattle}$	Probability that the threshold prevalence is reached within the *NoImpoCattle* herds on the day of testing
P_U	Within herd prevalence used either in the milking group only (testing strategies "a" and "b"), or overall in the herd (testing strategies "c" and "d")
$RR_{ImpoCattle}$	Relative risk of infection in the risk category *ImpoCattle*
$RR_{NoImpoCattle}$	Relative risk of infection in the risk category *NoImpoCattle*
Se	Test sensitivity
SerumCSe	Temporal sensitivity of the surveillance component based on individual serum testing (surveillance strategy "c" and "d")
Sp	Test specificity
SSe	Temporal surveillance system sensitivity
TI	Transiently infected cattle